Effect of light on Cephalosporium diospyri.

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EFFECT OF LIGHT ON CEPHALOSPORIUM DIOSPYRI.

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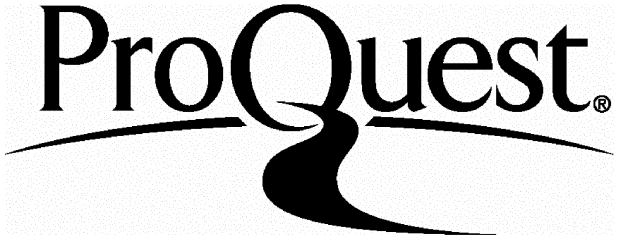

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SUMMARY

The process of carotenoid biosynthesis in C.diospyri was found to have an absolute requirement for light, and only light of a wavelength less than 500nm was effective for this photoinduction. An analysis of the carotenoid pigments of C.diospyri revealed that only phytoene was present in detectable amounts in dark grown cells. This might suggest that light was responsible for the expression of a hypothetical enzyme, phytoene dehydrogenase, which by a process of dehydrogenation was responsible for the appearance of the coloured carotenoids present in cells exposed to light.

A study of the mechanism of the photoinduction of carotenoids would indicate a process very similar to those described for N.crassa (Zalokar, 1954, 1955), Mycobacterium sp. (Rilling, 1961, 1964) and F.aquaeductuum (Eberhard et al, 1961), except that the production of carotenoids appeared to proceed at a much slower rate in C.diospyri. Three separate stages were recognisable. The initial photoinduction which, unlike the remainder of the process required light, was independent of temperature and required oxygen. The subsequent dark incubation consisted of a lag period before any carotenoids were synthesised followed by the actual production of the pigments. Both these latter stages were temperature dependent and also required oxygen.

Carotenoid synthesis in C.diospyri was inhibited by both diphenylamine and cyclohexamide, and only the stages after the initial photoinduction were sensitive to diphenylamine. Both PCMB and PHMB were found to substitute for light in carotenoid production in C.diospyri, but only when the fungus was grown on solid medium. No other -SH group inhibitor tested behaved as a photomimetic compound. In addition, antimycin A, found by Batra (1967) to have a similar property with Mycobacterium marinum

was ineffective against C.diospyri except that at higher concentrations inhibited both growth and carotenoid production.

Filtrates from dark grown cultures of C.diospyri were found to contain riboflavin with the corresponding production of lumichrome its acidic photolytic degradation product, in filtrates from light grown cultures. The physiology of the biosynthesis of riboflavin by C.diospyri appeared to resemble the process described by Kapŕalek (1962) for Eremothecium ashbyii, where cytochrome mediated respiration was replaced by a flavin type of respiration. Diphenylamine, but not cyclohexamide, in the same range of concentrations which inhibited carotenoid synthesis in light grown cells of C.diospyri also inhibited the extracellular production of riboflavin by dark grown cells.

No conclusive evidence was obtained which might suggest that light was responsible for the alteration of any other aspect of the metabolism of C.diospyri. Results would suggest that, in many areas of the metabolism, light and dark grown cells were the same. Parameters studied included sterol content of cells, the composition of their amino acid pools, their organic acid content, keto acids and soluble protein and isoenzyme composition.

The results obtained in this investigation are discussed in the light of other work and suggestions for possible lines of future research proposed.

INTRODUCTION

Although fungi are heterotrophic organisms, it has been known for many years that light induces in fungi a number of easily recognisable photoresponses. A non-critical guide to the earlier literature on general effects of light on fungi has been given by Marsh, Taylor and Bassler (1959). These photoresponses may be divided into two main categories: morphogenetic effects in which light induces or inhibits the formation of a structure, and non-morphogenetic effects, in which light influences the rate or direction of movement or growth of a structure, or the synthesis of a compound.

Non-morphogenetic responses of fungi to light may be further classified on the basis of the type of responding structure and the nature of the response. Both vegetative hyphae and reproductive structures respond to light. This response may be a non-orientated one in which either a stimulation or an inhibition of the rate of growth or the synthesis of the compound is the final expression of the effect, or an orientated response, where there is an altered spatial relationship of the fungus or part of the fungus to the source of illumination. This thesis considers particularly non-morphogentic non-orientated photoresponses, except in situations where examples of other types help to clarify or illustrate important concepts.

The Genus Cephalosporium

The genus Cephalosporium was established by Corda in 1839, based on the type species C. acremonium and subsequently over one hundred species have been placed in this genus. Most of these have, however, been

inadequately described and their relationship with other species are very ill-defined. Although placed with other Fungi Imperfecti, the perfect stage of a small number of cephalosporia has been distinguished, suggesting again the basis of the genus to be one of convenience of classification, rather than to illustrate affinities of members for a particular group. The genera Emericellopsis, Nectria, Allescheria and Cordyceps have all been reported as being the perfect stages of various cephalosporia.

In anatomical terms, this form genus is characterised by the production of oval to cylindrical spores of variable size and shape on simple conidiophores arising as short lateral branches from trailing hyphae. The spores are hyaline and non-septate, enclosed in a spherical ball of slime which is easily disrupted.

Many members of the genus have been reported (Pisano, 1963) to be associated with plant diseases, though whether causal or secondary infection has in many cases not been fully ascertained. Cephalosporium diospyri (A.T.C.C. 9066) was isolated by Crandall (1945), as causing wilt of Persimmon.

Non-orientated non-morphogenetic photoresponses.

Responses of this type exhibited by fungi are extremely varied. Examples of effects of light on the vegetative growth of fungi are conflicting and mostly non-critical, but in general terms, light is believed to depress vegetative growth in most fungi (Cochrane, 1958). The Zygomycete Syzigites megatocarpus, a fungus producing carotenoids in response to light, was found (Wenger & Lilly, 1966) to grow less

under continuous illumination, and this inhibition of growth was ascribed to the effect of light, not attempt being made to specify the quality of light responsible for this inhibition. As examples of the diverse effects light has on fungi, Diplodia zeae has been reported (Bemiller, Tegtmeier & Pappelis, 1968) to break down cellulose at a faster rate under illumination than in the dark, as well as producing two to four fold differences in fresh weight, but not dry weight. These results could be accountable possibly in differences between "cell bound" and "free" water of cells grown under the conditions of light and dark, but how light could bring this about has not been elucidated. Similarly, Duncan C.G. (1967) has shown that the decomposing activity of some wood decaying fungi was stimulated by light from the visible spectrum, especially shorter wavelengths.

The nature of the medium on which the fungus is grown seems in certain instances to determine whether light stimulates or depresses the growth rate. Both Hall (1933) and Dickson (1938) found that colonies of Sclerotinia fructigena on agar medium had a higher growth rate when exposed to light than if grown in complete darkness. Carlile (1965), also working with Sclerotinia fructigena however, found that under otherwise optimal conditions, the growth rate of cultures exposed to light was lower than those maintained in darkness and suggested that "staling", an ill-defined term embracing a wide range of changes in cultural conditions brought on by ageing of the culture, was more marked in dark grown than illuminated cultures.

When Penicillium claverigum was grown on malt extract agar, the growth rate was unaffected by light. If grown on Czapek Dox however, the growth rate as measured by colony diameter increase, was depressed by exposure to 24h, but not 12h light daily (Carlile, Dickens and Shipper, 1962). Trinci (1969) has critically studied growth of fungi, and the use of colony growth rate as a parameter, and has raised serious doubts as to its meaningfulness when used to compare growth rates of different species. He found that colony growth rate did not necessarily correlate with growth rates, measured by dry weight, in submerged cultures of the same fungi.

Nutritional conditions also appeared to influence the effect of light on the grown of Poria ambigua (Robbins & Hervey, 1960). They found the addition of yeast extract (500mgm/25ml) to the cultures resulted in an increase in dry weight in light grown but not in dark grown cultures. Yusef & Allam (1966), working with Pleurotus ostreatus, showed that the addition of either malt or yeast extract alone to basal medium did not affect growth, while a combination of malt and yeast extract exerted a growth promoting effect on cultures exposed to light and not significantly on cultures kept in darkness. However, Pleurotus dark grown cultures showed much higher dry weight values than light grown ones. These authors suggested that light favoured the breakdown of one or more substances in the medium beneficial for growth, and that it also exerted a drastic injury to cell metabolism.

Similar results were obtained by Weinhold & Hendrix (1963), who obtained evidence to suggest that light was responsible for the formation of peroxides in the medium, and it was these peroxides which

were inhibiting growth in light grown cultures of some fungal species. Hollomon (1966), revealed that light caused diminished growth of Phytophthora infestans on pea medium but did not consider peroxides as the immediate cause of this inhibition, although they were detectable in the medium. Rather, because of reversion of this effect by addition of reducing compounds, it was thought to be due to a decrease in reducing potential of the medium after illumination.

Static cultures of Phycomyces exposed to light continuously, produced lower final yields than those in the dark (Carlile, 1962). Mepacrine or lumichrome seemed to have differential effects in light and dark grown cultures which suggested differences in flavoprotein content between them. Hocking (1963) however, found this to be the case only in stationary cultures not in shake cultures, thus raising doubts as to the equivocality of the statement.

When Pilobolus kleineii was grown on synthetic media containing ferrichrome, light had no effect on the growth rate (Page 1956). If, however, haemin was substituted for ferrichrome, there was a marked depression of growth rate which was attributed directly to light and not shading of the hyphae by the haemin (Willoughby, 1961).

Two other environmental factors, hydrogen ion concentration and nitrogen source had an effect on mycelial growth of Cephalosporium sp. in liquid culture (Smith, 1960). Initial pH of 2.0, 7.2, and 9.4 promoted mycelial growth of the fungus in diurnal light, while initial pH of 5.2 and 9.9 promoted mycelial growth in continuous darkness. More mycelial growth occurred in diurnal light when the initial pH

was 2.6 and the nitrogen source was sodium nitrate. With ammonium chloride at pH 2.7, growth was inhibited by light and unaffected when yeast extract at pH 2.0 was used as a nitrogen source.

It has been suggested by Carlile (1965), that in many fungi there are significant metabolic differences between light and dark grown cultures, and that under optimal conditions, light metabolism and dark metabolism are equally effective in supporting growth. Departures from optimal conditions affect the processes differently. Another factor to be considered is that fungi may vary in their response to light, its quality and wavelength, the duration of exposure and the quantity or intensity. In many of the early conflicting reports, these factors were not deemed worthy of mention.

Relatively few instances have been reported in which light stimulates the growth of the fungus and which have not been later challenged. An example is in Thraustochytrium roseum (Goldstein, 1961), where up to 150% greater yields were obtained under 450 ft. candles, and this stimulation of growth was most pronounced during the early stages of logarithmic increase.

The only instance where the metabolic events subsequent to exposure of the fungal organism to light have been well documented is in a series of reports by Cantino and co-workers (1956, 1957, 1959, 1960, 1961, 1963, 1965 and 1966), working with the Phycomycete Blastocladiella emersonii. From any population of zoospores of B. emersonii, three different phenotypes were produced, the proportion of any one of them in the population depending on the nature of the medium and other

environmental conditions: 1. ordinary colourless organisms (O.C.), which did not produce an orange pigment, 2. orange organisms (O), which contained gamma carotene, and 3. resistant sporangial organisms, (R.S.), which possessed a brown thick pitted wall impregnated with melanin.

Almost all the enzyme activities associated with the T.C.A. cycle were demonstratable in cell free extracts of the O.C. organisms, but the significant factor was that, provided a minimum amount of CO_2 was available white light induced these colourless organisms to grow more rapidly, measured by an increase in mass, to fix CO_2 and consume glucose more rapidly, to accumulate a greater pool of soluble organic phosphorus, to consume glycine at a different rate, and to exhibit a greater ratio of D.N.A./total nucleic acid than they did in the dark.

Light in some unknown manner brought about increased CO_2 fixation, via reductive carboxylation of ketoglutarate to isocitrate by means of an NADP specific isocitrate dehydrogenase, cleavage of isocitrate to succinate and glyoxylate, transamination of glyoxylate with alanine to yield pyruvate and glycine, and finally, increased synthesis of thymine and/or thymidine, D.N.A., and nuclei as a result of the increased availability of glycine.

Cantino and Horenstein (1956), tentatively interpreted the effect of light in terms of a cyclic process involving succinate, ketoglutarate and isocitrate, the S.K.I. cycle (Fig. I) which they considered to be dominant to the T.C.A. cycle for the production of energy and intermediates in light grown cells. Light also stimulated polysaccharide

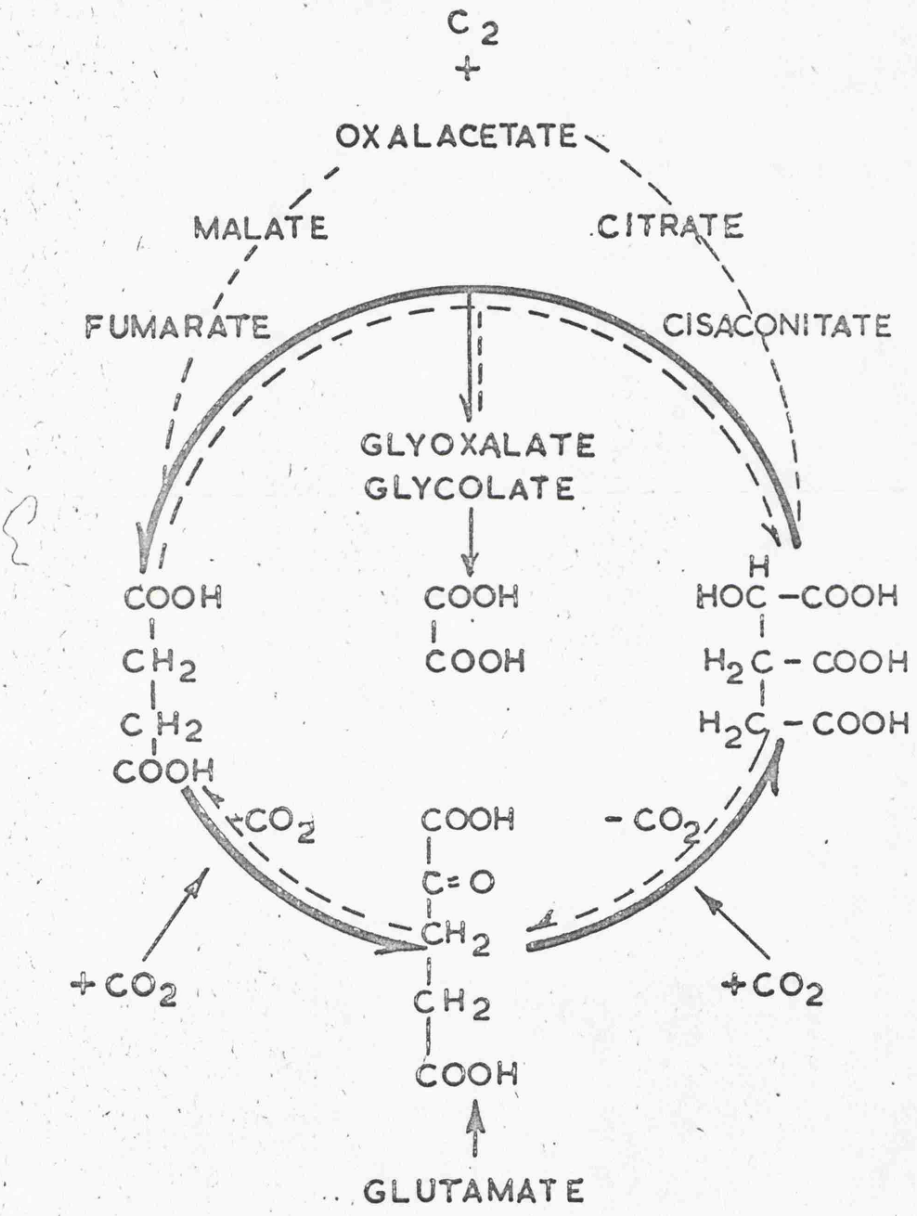


FIG. 1. The S.K.I. (succinate-ketoglutarate-isocitrate) cycle in Blastocladiella.

synthesis and reduced glucose-6-phosphate dehydrogenase activity (Goldstein & Cantino, 1962). Cantino has suggested that all these events were related, and could account for the increase in growth recorded for this organism as a direct response to light.

The photoreceptor has not yet been identified, nor has the precise locus of the light effect which accelerated carbon dioxide fixation, but it has been shown (Cantino & Turian, 1961) that the effective wavelengths were between 400nm and 500nm. Also in a later report, (Cantino, 1966) it was noted that light had altered the distribution of a haemoprotein resembling a cytochrome. In light grown cells, this compound was present in the soluble fraction, but in dark grown cells was bound to a pellet fraction. Whether this prophyrin had any direct correlation with photoreceptive action has not been determined.

Since this work with Blastocladiella emersonii, it has also been shown that light stimulated glucose uptake in Blastocladiella brittanica. (Cantino & Horenstein, 1964). Although in a closely related species of Blastocladiella, this observation suggests the possibility that general metabolism could be altered by light in many photosensitive fungi.

Action of Ultraviolet Radiation on Fungi.

Although it has been known since the work of Stevens (1928) that ultraviolet light, but not visible radiation, induced morphogenetic effects in some fungi, very little interest has been shown in non-morphogenetic effects of ultraviolet light. Goss & Frink (1934) reported the influence of ultraviolet radiation on the growth of a Cephalosporium. The longer exposures to ultraviolet inhibited the

growth of the fungus, measured as increase in diameter of the colony, and therefore of doubtful validity, and caused the colonies to become dark brown; the fungus was not killed nor was sporulation materially altered.

Ultraviolet rays induce many fungi to sporulate, as shown by Leach (1962) with Helminthosporium oryzae. In Verticillium alboatrum, ultraviolet radiation has been shown to inhibit the production of melanin and microsclerotia, and also influence growth (Brandt, 1964). A diffusable morphogenetic factor stimulating the production of melanin and microsclerotia has been discovered, and the suppression of the synthesis of this compound by ultraviolet radiation demonstrated.

Influence of light on Pigmentation in Fungi.

Many non-photosynthetic microrganisms, including some fungi, produce pigments in response to light, the commonest being carotenoids, resulting in easily distinguishable colour differences between organisms grown in the light and those grown in the dark. Absolute requirements for light have been demonstrated in many fungi including Pyronema confluens (Carlile & Friend, 1956), Dacromyces sp. (Bulat, 1954) and Fusarium aquaeductuum (Eberhard, Rau & Zehender, 1960). The pink and orange pigmentation in cultures of various members of the from genus Cephalosporium has been noted by several authors. However, only a few, eg. (Janke, 1949) have mentioned briefly the influence of light on colour formation in this form genus, and without attaching any significance to their observations. Codner & Platt (1959), have examined 75 Cephalosporia, and have revealed that this form genus could be divided into three groups depending on the colour production in response to light.

Group I: pigment not produced in either light or dark grown cultures.

Group II. pigment only produced in light grown cultures

Group III. pigment produced in both light and dark grown cultures

In some organisms light merely stimulates carotenoid formation as in Phycomyces blakesleeanus (Garton, Goodwin & Lizinsky, 1951), Penicillium sclerotium (Mase et al, 1957), Karlingea rosea (Haskins & Weston, 1950). Goldstrohm & Lilly (1965), found in Dacryopinax spathularia, that light grown cells of seven isolates produced carotenoids, and in a later report (Vail & Lilly, 1968), further revealed these pigments were concentrated at the cell wall or the plasma membrane, and light grown cells produced eight times as much pigments as dark grown cells. Interestingly, also, the cell walls of light grown cells were between 1.5 and 1.9 times the thickness of dark grown cells.

In Neurospora crassa, the conidia contained carotenoids even when the fungus was grown in darkness, but carotenoid biosynthesis by the mycelium required light. It was shown by Zalokar (1954), and Harding, Huang & Mitchell (1968) that there was an accumulation of the colourless more saturated polyenes in the mycelium in darkness, and their conversion by dehydrogenation to the relatively unsaturated coloured carotenoids required both light and oxygen. Exposure to light for as little as one minute allowed the mycelium, when placed in dark under aerobic conditions to synthesise carotenoids almost to the level of that produced under illumination.

Very similar results have been found with Fusarium aquaeductuum (Rau, 1961), Fusarium oxysporum (Carlile, 1956), and the bacterium

Mycobacterium marinum and Mycobacterium sp. (Matthew, 1963; Rilling, 1961). This photoinduction of pigment has been shown in the organisms so far studied to exhibit a number of common features, resulting from an initial photooxidation of a compound or compounds as yet unidentified, followed by a period of protein synthesis and then finally carotenoid formation. In the photoinduction of carotenoid synthesis in the sporangiophore of Aspergillus giganteus, the initial photochemical step appeared to be unusual in that oxygen was not essential (Trinci & Banbury, 1969).

Contrary to the above observations, it has been reported that light grown cultures of a mutant of Blastocladiella emersonii contained less carotenoid than those grown in darkness (Cantino & Horenstein, 1956), though whether light was inhibiting carotenoid synthesis, or they were destroyed in vivo by light has not been revealed. Similarly, in Epicoccum nigrum, light (2600 lux) inhibited carotenoid synthesis at 24°, but was stimulating at 28° (Gribanowski - Sassu & Foppen, 1968). Ergosterol synthesis was also effected, but there was no evidence of correlation between carotenoid and sterol synthesis although both share common biosynthetic steps. This apparent temperature sensitivity of pigment synthesis could be explained in that mycelial growth at 24° was not dependent on light, whereas at 28° light had a stimulating effect on growth.

As carotenoids are not generally believed to be primary photoreceptors in fungi, their function has been open to speculation. It has been shown by Goldstrohm & Lilly (1965) that carotenoids

protected Dacryophinax spathularia against light, and Stanier (1960) has suggested a similar function in some photosynthetic bacteria. However many fungi produce carotenoids without any exposure to light. Mackinney & Chichester (1960) have suggested the possibility of a detoxification mechanism for unwanted hydrocarbons resulting in the formation of carotenoids, but it is difficult to see how this would be conceivable, since in the majority of fungi, with carotenoids produced in such small amounts, a minor proportion of the metabolic turnover would be involved in their synthesis.

The synthesis of pigments other than carotenoids have also been shown to be influenced by light. In Penicillium herquei (Riedhart & Porter, 1958), cultures grown in the dark produced a yellow pigment which underwent a reversible photooxidation to a green form when illuminated. The pigment which was very sensitive to H^+ was probably acting as a pH indicator, changing colour in response to intracellular changes in pH caused by illumination. As well as initiating carotenoid formation, light depressed naphthoquinone production in Fusarium oxysporum (Carlile, 1956). Melanin formation was stimulated by light in Aureobasidium pullulans (Lingappa et al, 1963), but was inhibited in a black mutant of Neurospora crassa (Schaeffer, 1953), once again illustrating the extreme variation in the effects that light has on fungi, and the many different forms in which the end result of the effect may be manifested.

The Nature and Mechanism of the Photoresponse.

Carlile (1965) states that 'one of the most important steps in the

elucidation of the biochemical basis of a photoresponse is the establishment of the chemical nature of the photoreceptor'. The approach to this problem has varied, but the determination of the action spectrum of the response has been used in many cases. The relative effectiveness of different wavelengths in producing the photoresponse is determined, since the action spectrum of the photoresponse should closely parallel the absorption spectrum of the photoreceptor compound. The action spectrum is constructed on an equal response basis, where the dosage required at each wavelength to give a standard response is determined, and its reciprocal plotted, keeping the exposure time constant and varying the light response.

The most detailed action spectrum available for fungal photoresponses have been published by Delbrück & Shropshire (1960) for phototropism and the light growth reaction in the sporangiophore of Phycomyces, and by Curry & Gruen (1959) for phototropism in the same species. Their results were in good agreement (Table 1). Curry & Gruen interpreted theirs as favouring the hypothesis of a carotenoid photoreceptor, whereas Delbrück & Shropshire, because of the peak in the ultraviolet region, excluded β-carotene in favour of a flavin compound.

Data suggesting an action spectrum similar to that obtained for Phycomyces have been reported for the stimulation of growth in Blastocladiella emersonii (Cantino & Turian, 1961), trophocyst formation in Pilobolus kleinei (Page, 1956), the induction of caro-

TABLE I

Some action spectra for Photoresponses in Fungi & Bacteria.

Authors	Organism	Type of Response	Wavelength Max.	Suggested Photoreceptor
Delbruck & Shropshire	Phycomyces	Phototropism	280 385 455 485	Flavin
Curry & Gruen	Phycomyces	Phototropism	280 370 445 470	Carotenoid
Cantino & Horenstein	B.emersonii	Stimulation of growth.	400 500	No decision
Page	Pilobolus kleinei	Trophocyst formation	410 420.50 480	Flavin
Zalokar	Neurospora crassa	Carotenogenesis	450 480	Flavin
Rau	Fusarium Aquaeductuum	"	375/380 450/455 470/480	Flavin
Rilling	Mycobacterium sp.	"	375 425-480	Flavin
	Mycobacterium marinum	"	404 493 577	Porphyrin
Burchard & Hendricks	Myxococcus xanthus	"	405.410 512 585 635	Protoporphyrin IX
Kumagai & Oda	Trichoderma viride	Sporulation	320 380 430 480	Not Flavin possibly carotenoid
	Riboflavin in 0.1m pH7 phos. buffer		266 373 445	
	β-carotene in hexane		451 482	

tenoid biosynthesis in Neurospora crassa (Zalokar, 1954), Fusarium aquaeductuum (Rau, 1967) Mycobacterium sp. (Rilling, 1964) and many other photobiological effects on fungi. (Table I).

Thus, the similarity of the detailed action spectra as well as the spectral limits determined for those responses suggest that one pigment, or class of pigments functions as the photoreceptor in the majority of photoresponses. A possible exception has been reported in Entomophthora coronata (Page & Brungard, 1961) where response was obtained for conidia phototropism to wavelengths up to 630nm, although with much less sensitivity than at 405nm. This organism contained no identifiable carotenoids, but did contain a pigment characteristic of a porphyrin with absorption peaks at 412, 505, 543, 580 and 630nm. Also sporulation in Trichoderma viride, although responding to light from the blue end of the spectrum appears to have an action spectrum for the photoresponse uncharacteristic of a flavoprotein (Kumagai & Oda, 1969), as does photoinduction of carotenogenesis in Mycobacterium marinum (Rilling, 1964), and Myxococcus xanthus. (Burchard & Hendricks, 1969).

Cases of sensitivity of fungi to red light have been reported. Fruit body production in Sphaerobolus stellatus was dependent on light of wavelengths less than 520nm, but only light of wavelength longer than 520nm was effective in promoting diurnal periodicity of spore discharge (Alasoadura, 1963). Alternaria solani has also been reported to respond to red light (Lukens, 1963). These observations do not rule out the universality of a flavoprotein receptor, since Mahler (1954) showed that the presence of copper in the flavoprotein butyryl CoA dehydrogenase

resulted in a spectral shift of activity to the red end of the spectrum. However, cases have been reported where a second class of photoreceptor has been indicated, as in Neurospora crassa (Klein & Klein, 1962) where far red light augmented the lethal effects of X-ray irradiation of the conidia, which in turn was negated by subsequent exposure to red light.

Action spectra have been obtained for photomorphogenesis in some Fungi Imperfecti and Ascomycetes in which ultra violet radiation appears to be responsible alone. Leach (1962) for example found that exposure to monochromatic radiation at 238, 254, 280 and 313nm were effective in stimulating vegetative repoduction Pleospora herbarum. These results, and a recent review by Trione & Leach (1969) cites many examples of light dependent sporogenic substances in fungi, suggest the widespread occurrence of a photoreceptor substance or substances with an absorption spectrum wholly in the ultraviolet region. Isolates from a wide range of fungi contained a substance having maximum absorption at 310nm in water extracts of mycelium exposed to ultraviolet radiation, but absent in dark grown cells (Leach, 1965). The absorption curve was similar to that of a pyrimidine dimer, though it has not been positively identified.

In many reports, workers in deciding as to whether a particular compound could be acting as the photoreceptor have tried to correlate its concentration with the photosensitivity of the organism under investigation. This approach has been used more frequently in testing the carotenoid rather than the flavin hypothesis, mainly because

flavins are known to be widespread in distribution and function, whereas the distribution of carotenoids is much more restricted, and their function more open to speculation.

Carotenoids have not been detected in the photo-sensitive stipes of Collybia velutipes (Aschan - Aberg, 1960), and an albino mutant of Pyronema confluens (Carlile et al, 1961) produced apothecia as readily when exposed to light as did the pigmented wild type. Diphenylamine, an inhibitor of carotenoid synthesis, had no effect on trophocyst formation in Pilobolus kleinei (Page, 1956), nor did a diphenylamine treated culture of Phycomyces with less than 3% of the normal carotenoid content lack phototropism (Goodwin, 1952). This might suggest that the hypothesis of carotenoids as photoreceptors has little experimental support. However, the sensitivity of the photoinduced sporulation of Trichoderma viride decreased when the organism was cultured on media supplemented with diphenylamine at concentrations higher than 6×10^{-6}M. (Kumagai & Oda, 1969). The fact that DNA is known to react with diphenylamine (Slater 1961), and therefore, is quite capable of interfering with cell metabolism at this basic level could be held against these arguments for a carotenoid photoreceptor. Nevertheless the action spectrum obtained for the photoresponse in Trichoderma viride was not characteristic of a flavoprotein.

Similar work has been reported using competitive inhibitors of riboflavin in in vivo experiments. It has been found for instance, that lyxoflavin inhibited light induced trophocyst formation in Pilobolus kleinei, and this inhibition was reversed by addition of

riboflavin (Page, 1956). In Hypomyces solani mepacrine inhibited formation of perithecium formation, and riboflavin was again antagonistic. Tschabold, (1967), suggested this might be the photoreceptor substance, but many euthors e.g. Hemker & Hulsmann, (1960) have raised objections to mepacrine as a specific inhibitor of flavoprotein synthesis and for this reason also, the report that mepacrine had differential effects on the growth of Phycomyces in light and dark due to differences in flavo-protein content (Carlile 1962), must also be regarded as doubtful, and in need of further study.

Lukens (1963) has reported that the addition of flavin monoucleotide partially overcame the inhibitory effect of light exposure on sporulation in Alternaria solani, and Carlile (1965) found with Sclerotinia fructigena that 1,2 dichloro 4-5-diaminobenzene, another inhibitor of flavin synthesis, inhibited growth of light grown cultures to a greater extent than dark grown cells. Recently, Hart and Filner (1967) used the specific photodestruction of flavin by phenyl acetic acid (Hemmerich, Massey and Webber 1967), and reported that this compound appeared to be a relatively specific inhibitor of phototrophic curvature in Avena coleoptiles. They suggested that these results supported the hypothesis that the flavin moiety was involved in the reception of the light signal. The fact that this compound as well as mepacrine, was without effect on photoinduced sporulation in Trichoderma viride (Kumagai & Oda 1969) where carotenoids have been implicated in the photo-response, adds weight to the flavin hypothesis operating in Avena.

Further evidence comes from an early observation by Hopkins (1937) that

riboflavin would catalyse the photooxidation of ascorbic acid, and later work extended this observation to the photooxidation of indole-3-acetic acid (Galston 1949). Vernon (1959) showed that illuminated flavin mononucleotide mediated the oxidation of NADH, with a concomitant reduction in cytochrome C, and initiation of activity in an electron transport path by illumination of a flavoprotein has been demonstrated by Lewis, Schiff and Epstein (1961), in Euglena. Although these experiments were not carried out on fungal tissue, they do show that flavoproteins on exposure to light could alter electron transport mechanisms. Therefore in many reported cases, evidence has been produced which would appear to favour the existence of a flavoprotein as the photoreceptor compound in fungi. Recently however, Thornton (1969) found in the sporangiophores of Phycomyces, "crystalloids" consisting mainly of acidic protein material. Dark grown sporangiophores were consistently found to contain two to four times as much crystalloid material as light grown sporangiophores, and the author suggested that these may play some role in the mechanism of light reaction in other fungi known to contain them.

In some photoresponses there appears to be a definite light-sterol relationship, as in the case reported by Sproston & Setlow (1968) who found that ergosterol substituted for ultraviolet light in the sporulation of Stemphyllium solani. The role of steroids in photo-responses have been interpreted by Narasimhulu & Rosenthal (1964) as involving the interaction between an unidentified oxidation - reduction component and a hydroxylating enzyme of steroids. In the

absence of NADPH or steroid, the -SH group of the enzyme is bound to an oxidation reduction component X, to form a complex S:XH. When steroid is added, X is free to participate in electron transport from NADPH to oxygen by way of the co-sensitive oxygen activating pigment. This hypothesis could help to explain many membrane bound light sensitive systems in fungi, as well as allowing for the alternative electron transport pathway postulated in other photoresponses.

Although metabolic events associated with the photoresponse in fungi have only been described in B. emersonii (Cantino & Horenstein 1956), there have been other reports where light appears to have altered metabolism other than that obviously manifested in the photoresponse. Carlile (1965) states that dark grown cultures of Sclerotinia fructigena gave a strong Nadi reaction unlike light grown ones. This test used to indicate cytochrome oxidase activity might, therefore, be taken to suggest an alternative electron transport pathway, but objections to the specificty of the Nadi reaction have been raised by Maehly (1954), who found that the test was positive even in the absence of any oxidising enzyme, thus raising doubts as to the importance of the above finding. Another report suggesting alternative electron transport was that of Hall (1967) who found altered concentration of catalase isoenzymes in light and dark grown cultures of Fusarium solani.

Light grown cells of Cladosporium mansonii were found by Sussman et al (1963) to be predominantly mycelial, whereas dark grown cells were

unicellular and much larger. Thus it would appear that light had altered cell wall synthesis in some way, as appears to be the case with Dacryopinax spathularia (Vail & Lilly 1967), where cell walls of light grown cells were 1.5 to 1.9 times the thickness of those found in dark grown cells.

In a recent study on the sporangiophore of Phycomyces, it was reported (Gettens & Shropshire 1963) that the concentration of reducing sugar and a hitherto unknown flavin, paralleled the course of transient photoresponse. These workers also reported that the concentration of ATP increased after the sporangiophores had been exposed to a saturated amount of light. The lack of knowledge in this field obviates the need for further study.

Some effects of the action of Photomimetic Compounds on Fungi and Bacteria.

Since Charlton (1953) discovered that light could be substituted for by hydrogen peroxide in the sporulation of Alternaria solani, and the observation of Turian & Cantino (1959), that they could imitate the effect of light on B. emersonii by supplying succinate and glyoxylate to dark grown cells, other examples of photomimetic compounds have been recently studied.

Rau (1967) reported that in the light dependent carotenogenesis in Fusarium aquaeductuum, the photo induction could be substituted by incubation of the mycelium with the specific-SH group inhibitors, p-chloro and p-hydroxymercuribenzoate (PCMB & PHMB) in the dark. He originally concluded that light and mercuribenzoate catalysed the same

reaction in the chain of regulatory mechanism by blocking -SH groups, and postulated several possible flavoproteins having similar action spectra to the photoresponse. In a later publication however, Thiemer & Rau (1969) demonstrated with mutants of F. aquaeductuum lacking photoregulation of carotenoid synthesis, that in contrast to the earlier hypothesis, the sites of action of light and mercuribenzoate were different, since their effects were additive.

Shortly afterwards, a report by Batra (1967) showed that Antimycin A, an antibiotic specific for inhibition of cytochrome b reductase, induced the synthesis of carotenoids in Mycobacterium marinum in the absence of light. He found Antimycin A to be 300-400% more effective than saturating amounts of light in the induction of carotenoid synthesis, and, like the photomimetic compounds used by Rau, the effects of light and Antimycin A were additive. PCMB and PHMB inhibited rather than stimulated carotenogenesis in M. marinum (Batra, Gleason & Jenkins, 1969), suggesting different modes of action for them as photomimetic compounds.

Since, in both cases these compounds could be negated by the subsequent addition of known inhibitors of protein synthesis to the mycelium (cyclohexamide with F. aquaeductuum, and chloramphenicol with M. marinum), it appears that they may be acting by inhibiting the action of a repressor, allowing subsequent de novo synthesis of the proteins required for carotenogenesis.

Because Cephalosporium diospyri, the organism used in this investigation was obviously light sensitive in that it produced carotenoid pigments

in response to light, and because there has been no report on the mechanism of photoinduction of these pigments in this organism, this investigation was undertaken in the hope that such information might be obtained. It was also decided to investigate the possibility of altered general metabolism due to light in this fungus because of the lack of knowledge in this very neglected field of photobiology.

MATERIALS AND METHODS

(a). Growth and Maintenance of Cultures.

The organism Cephalosporium diospyri (ATCC 9066) was first isolated and described by Crandall (1945) as causing wilt of Persimmon, and for this investigation was reclaimed from a freeze dried culture from the Bath University of Technology culture collection.

Cultures were maintained in sterile soil kept at 4° and an inoculation procedure was adopted as standard which would provide large spore crops needed to inoculate the number of flasks required for any one experiment. This consisted of a primary inoculum grown on malt extract agar slopes in loz universal bottles (2% Oxoid malt extract; 0.5% Oxoid mycological peptone; 1.5% Oxoid agar) for seven days at 25°. Spores from these cultures obtained by scraping the mat of mycelium in 5ml distilled water were used to inoculate seed flasks of a chemically defined medium described by Schopfer (1934) and consisting of :-

7% Glucose
0.2% Glycine
0.15% Potassium dihydrogen phosphate
0.05% Magnesium sulphate
0.05% Oxoid Yeast Extract

50ml amounts in 250ml conical flasks were autoclaved at 10lb/sq.in. for 10 min, after which no caramelisation had occurred, and the pH was 4.8. Yeast extract was included in the medium as the provision of growth factors appeared to improve final growth yields. After growth for seven days (see later for details), the cells were harvested aseptically by centrifugation at 6000rpm for 30min on an M.S.E. High

Speed 18 centrifuge, washed twice with sterile distilled water and finally suspended in distilled water.

To obtain a uniform spore suspension, and to remove mycelial fragments and clumps, this suspension was then filtered aseptically through glass wool, and the optical density at 610nm was adjusted so that a one in ten dilution gave an optical density of one. All the aforementioned manipulations and growing stages were carried out in darkness.

The medium used in most experiments was that described above. In some experiments, the organism was grown on 2% Malt Extract Broth (Oxoid CM 57) and Czapek Dox Broth (Oxoid CM 95). Cultures were grown on Gallenkamp Orbital incubators at 25^{o} using a 1" throw at 250 rev/min. In order to compare the growth of submerged cultures of C.diospyri in light and darkness, two Gallenkamp Orbital incubators were used. The light grown organisms were continuously illuminated directly from above by four 40 watt fluorescent tubes (Ecko white light), in a white box. The light path was 45cm and yielded approximately 2000ft candles at the surface of the cultures. Circulation of cold air between the cultures and the light source, achieved by placing the incubators in a ventilated cold place, maintained the temperature at 25^{o}.

"Light tightness" of the incubator used to grow cells in complete darkness was tested by exposing a photographic film inside the blacked out incubator over seven days. All comparative experiments were carried out from the same inoculum, thus ensuring that any differences

in cells would be solely to the effects of light and not from any variation in inoculum, medium composition or other physical growth conditions.

Cultures were harvested by centrifugation at 6000rpm for 20min. at 5° on an M.S.E. 18 centrifuge, washed with distilled water twice and freeze dried. Dried cells were then finely ground to a powder in a pestle and mortar, and stored at -20°. Culture filtrates were kept frozen until needed.

Experiments were also carried out in Petri dishes using either 2% Malt Extract agar or Schopfer's medium containing 2% Oxoid agar. The inoculum used in these experiments was obtained from the primary inoculum grown on malt agar slopes. Petri dish cultures were grown under continuous illumination of approximately 2000ft candles or in complete darkness for the required times.

(b). Preparation of Cell Free extracts for Analytical Purposes.

In order to obtain extracts of the fungus for analysis, several methods of disruption were assessed for their efficiency in breaking cells of C. diospyri. Although vigorous grinding of mycelium in a pestle and mortar with an abrasive such as sand or carborundum has been widely used as a method for preparing cell extracts in fungi, (eg. Boulter & Hurst, 1960) this technique was unsuitable, as were other conventional homogenising methods for C.diospyri. The thickness and stickiness due to copious secretion of large polymer material of the leathery hyphae, rendered homogenisation in a pestle with abrasive slow and, furthermore,

cells were seldom broken (approx. 5% after 15min, determined microscopically). Potter-Elveheim glass homogenisers were a slight improvement, but not suitable for large amounts of cells. Ultrasonic disintegration using an M.S.E. 50 sonic oscillator gave up to 20% disruption after 15 min, slightly less than that obtained with a French Pressure Cell.

None of the previous methods gave results as satisfactory as those obtained using a Braun homogeniser (Shandon & Co. Ltd., London). 1gm mycelium in 19ml of a suitable liquid were placed in a Braun homogeniser bottle, capacity 75 ml, containing 50gm 0.45-0.50mm diameter Ballotini glass beads. The sample was homogenised for 4 min at maximum revolutions (4000 rpm) in a sufficient stream of carbon dioxide to keep the sample near freezing without actually becoming solid. After homogenisation which gave a consistent break of 75-80%, determined visually, the beads were removed by filtration on a No.1 porosity sintered glass funnel and the filtrate centrifuged at 18000rpm (38,000g) on an M.S.E. High Speed centrifuge at 4^{o} for 30 min.

(c). Separation of Soluble Amino Acid Pools.

Weighed portions of finely powdered mycelium (500 mgm) were exhaustively extracted in a Soxhlet apparatus with light petroleum (b.p. 40-60) to remove lipid material found to interfere with subsequent chromatography of amino acids. The defatted fungal tissue was then carefully removed from the extraction glass sinters and dried at room temperature. For the extraction of amino acid pools from the fungus,

various extracting procedures were tried in preliminary experiments, and it was decided for this investigation that extraction of the tissue with hot 60% v/v aqueous ethanol three times for 1h was sufficient to remove all ninhydrin positive material. 50ml extraction solution was used for every 200mg dry weight of fungus.

The extracts were combined and concentrated to dryness on a Buchii rotary evaporator at 50° under reduced pressure, and extracted with 50ml of the ethanol and water and concentrated hydrochloric acid (95 + 4.5 + 0.5 v/v) desalting mixture described by Baliga et al (1955). This extract was concentrated and placed at -20° overnight to facilitate the removal of salt, filtered, and the amino acids separated and identified by descending one way paper chromatography using as the solvent the butan-1-ol + acetic acid + 96% (v/v) ethanol + water (40 + 10 + 10 + 20 v/v) mixture on Whatman No.1 filter paper. After chromatography until the solvent front was about 2cm from the end of the papers, these were dried and sprayed with the alkaline ninhydrin spray described by Kay, Harris Entenman (1956), which was found to be much more sensitive that polychromatic ninhydrin. Amino acids were identified from known maps separated in the same manner. This method was found to be more satisfactory than those separation methods employing thin layer chromatography.

(d) Extraction and Separation of Organic Acids.

(i) Extraction.

Finely powdered tissue was extracted in 60% (v/v aqueous ethanol in the Braun homogeniser as described earlier. Because of failure

to detect organic acids in extracts from 250mg dry weight portions of fungal cells, the starting material was increased to 1g dry weight. After extraction, the cell debris was removed by centrifugation, washed with a further 50ml of 60% (v/v) ethanol, the extracts combined and the ethanol removed by evaporation under reduced pressure on a Buchii rotary evaporator at room temperature. The aqueous extract was then concentrated and purified by passage through Dowex 50 (H^+ form) and Amberlite IRA 400 (CO_3^{--}) ion exchange resin columns, as follows.

(ii) Ion Exchange Columns.

A modification of the method of Bryant & Overell (1953) was used. Dowex 50 (H^+) was prepared by successively washing with 2N sodium hydroxide, 2N hydrochloric acid and water, in that order, for several cycles until all colouring matter had been removed. Columns (20cm x 1cm internal diameter) were packed wet with resin to a depth of 15cm, and after use, regenerated with 200ml 2N hydrochloric acid followed by water until the pH of the effluent was neutral. About 20g of Amberlite IRA 400 ($C1^-$), as from the bottle, was exchanged with ca. 500ml 2N sodium carbonate overnight, and columns (1cm internal diameter) were packed wet to a depth of 15cm. After passage through the columns of another 500ml 2N sodium carbonate until the effluent was free of $C1^-$ (tested with a saturated solution of silver nitrate in conc. nitric acid), 500ml of water was passed through to wash out all excess alkali, and the columns were then ready for use. Regeneration was achieved with 500ml 2N sodium

carbonate and a final washing with distilled water until the washings were at neutral pH.

(iii) Application and Chromatography of samples.

Aqueous samples in a final volume of less than 5ml were carefully applied to the Dowex 50 (H^+) columns which retained amino acids, and washed through with 100ml glass distilled water directly on to the Amberlite IRA 400 anion exchange columns. Sugars and non acidic material passed through both columns while the organic acids were retained on the anion exchange columns, and were eluted from them with 100ml 2N ammonium carbonate (72g litre). The ammonium carbonate was removed by repeatedly taking the samples to dryness under reduced pressure on a water jet pump and redissolving the residue in distilled water. Care was taken to ensure that the evaporating temperature was less than 40°. Finally the samples were dissolved in 1ml distilled water ready for chromatography. Chromatographic separation of organic acids was carried out by the method of Lugg and Overell (1948). Samples 20 µl, were spotted on to Whatman No. 1 paper and run in descending direction using the solvent system, butan-1-ol + formic acid + water (4 + 1 + 5 v/v) a diphasic solvent system, the lower phase being removed before use and placed in the tank. Organic acids were identified from known maps treated in exactly the same manner. The location reagents used in early experiments were aniline xylose and bromocresol purple, but abandoned in favour of the more sensitive dimethyl-glyoxime nickel biuret reagent described by Savoury (1964), where

acids appeared as pink spots on a white background. This reagent also had the advantage that colour could be stabilised by removing excess reagents from the paper by using an ethanol ammonia wash after spraying.

(e) Extraction and separation of Keto Acids.

A combination of the methods described by Hulme (1961) and Cavallini & Mondovi (1957) were employed. All stages in the extraction were performed at 0°.

Samples (1g dry weight) were homogenised at 0° in the Braun homogeniser in 5% metaphosphoric acid, the cell debris removed by centrifugation at 38,000g at 0° for 30 min. 2ml 0.2% 2,4-dinitrophenyl hydrazine in 2N HCl was added to the extract, and after 30 min incubation at room temperature, three drops of formaldehyde were added to avoid artefact formation. Colour was extracted into a total of 15ml diethyl ether in 3 x 5 ml portions. After removal of the ether by evaporation, 1ml 1N ammonia solution was added to dissolve the keto acid derivatives. Finally, 1ml chloroform was added to remove unreacted excess DNP, the mixture vigorously shaken, centrifuged and the ammonia layer removed for chromatography.

An aliquot (10 μl) of this solution was applied to a sheet of Whatman No.1 paper and run in ascending direction in the solvent butan-1-ol + ethanol + water (4 + 1 + 5 v/v). Keto acids were detected as their DNP derivatives by direct vision or under a U.V. lamp (Camag TL 900) at 254nm and identified by running DNP derivatives of authentic samples prepared in the same fashion, and subsequent co-chromatography of the test samples

with them.

(f) Electrophoresis of extracts on Polyacrylamide Gels.

Polyacrylamide gels were formed in glass tubes (internal bore of 5mm) previously cleaned and rinsed in 0.5% aqueous solution of PhotoFlo (Kodak, Ltd).

1ml of a 7.5% acrylamide solution pH 9.5, was used for the separating gel which was allowed to polymerise before 0.2ml of the stacking gel, pH 8.9, was run on to the top. Both were prepared as described by Davies (1964). After polymerisation, the tubes were placed in the electrophoresis apparatus which consisted of two rectangular Perspex tanks placed one above the other (Laycock, Thurman & Boulter, 1965). The tubes were mounted vertically, connecting each end with the reservoir buffer solution (0.1M Tris (trihydroxymethyl) methylamine) - glycine buffer, pH 8.3 in such a way that the lower solution was the anode, and the upper one the cathode. Hence, proteins as anions separate as they move downwards through the separating gels after a preliminary concentration at the large pore: small pore interface.

A suitable volume of cell extract (less than 0.1ml) containing 250 ug protein and prepared by homogenising 1g dry weight of cells in 19ml 0.1M Tris buffer solution pH 8.0, containing 170g/litre sucrose, 1g/litre ascorbate, 1g/litre L-cysteine hydrochloride (Stahmann, 1963), was applied to the top of each tube. Bromophenol blue, a dye, was added to the buffer in the top tank to indicate visibly the position of the moving front. Effective separation of protein was obtained by using a constant current of 2.5m amp per gel column supplied by a Vokam 80mA 400 volt

power supply. Electrophoresis carried out at room temperature was continued until the marker dye had migrated to within 2mm of the base of the tubes.

After electrophoresis, the gels were removed from the tubes by gently loosening them from the glass with a hypodermic needle through which distilled water flowed.

Staining of gels.

(a) Protein (Davies, 1964).

Total protein was detected by immersing gels in a solution of 1% naphthalene black 12B in 20% acetic acid, and excess dye removed by de-staining in 7% acetic acid.

(b) Esterases (Lawrence et al, 1960).

Esterase isoenzyme were detected by their ability to liberate α naphthol at the site of activity within the gels by cleavage of the ester linkage of α naphthyl acetate on butyrate. The free α naphthol is coupled with a diazo dye, Fast Blue BB (Sigma) to produce an intense brown colour. Gels were placed in 50ml 0.1M Tris-maleate buffer, pH 6.4, containing 50mg Fast Blue BB, 1ml α naphthyl acetate or 1% α naphthyl butyrate in 50% acetone for $1\frac{1}{2}$h at room temperature.

(c) Phosphatases

Acid phosphatases were detected by immersion of gels in the incubation solution suggested by Rudolph & Stahmann (1966). This consisted of 50ml 0.1M acetate buffer, pH 4,0, containing 50mg Diazo Blue B (Sigma), and 50mg α naphthyl phosphate. Alkaline phosphatases were detected by

using the same incubation solution as above, except that 50ml 0.1M Tris - chloride buffer, pH 9.2, was substituted for the acid buffer.

In both these reactions, the Diazo dye couples with free naphthol released by action of the enzyme to give a red colour.

(d) Catalases and Peroxidases.

To detect catalase activity, the gels were soaked in 0.2M aqueous catechol solution for 30 min, washed in distilled water and soaked in 0.3% hydrogen peroxide solution for 5 min until brown bands developed on a white background. Peroxidase activity was tested for by substituting 0.2M guiacol for catechol in the incubation. No bands were detected in any experiments, and by the criteria of Rudolph & Stahmann (1964), this observation suggests that the enzymes detected with the catechol were catalases and not peroxidases.

Recording of Results.

Gels stained for protein and isoenzyme activity were stored in small glass polythene capped bottles. Densitometer traces of gels were obtained by scanning them in a Joyce-Loebl Chromoscan (Joyce-Loebl Ltd., Gateshead), using appropriate wedges, filters and slit sizes. In all comparative experiments, the same conditions for scanning were used. Although most isoenzymes gave stable colour reactions, those showing catalase activity were scanned within 10min of staining to minimise diffusion of colour and formation of gas bubbles in the gels formed from the breakdown of hydrogen peroxide.

(g) Chemical Fractionation of Mycelium.

Freeze dried cells were fractionated by a modification of the procedure used by Roberts et al (1955), with E.coli.

(i) 100mg cells were suspended in 16ml 5% TCA at 5° and shaken for 1h. This extraction was repeated, the supernatants combined, extracted three times with diethyl ether to remove the T.C.A., and analysed for nitrogen, Phosphorus and total carbohydrate.

(ii) The residue was then suspended in 16ml 75% ethanol for 30 min at 50° and centrifuged.

(iii) Then the residue from (ii) was suspended in 16ml methanol: chloroform (1 : 2) at 50° for 1h and centrifuged. The supernatant was combined with that from (ii).

(iv) Finally, the residue from (iii) was suspended in 16ml 5% T.C.A. in a boiling water bath for 30 min. This extraction was repeated and the combined extracts analyses for RNA and DNA.

Supernatants from (ii) and (iii) were combined with 70ml distilled water and 70ml ether. The aqueous phase was further extracted three times with 70ml ether, the ether fractions combined, reduced to dryness and weighed to give the lipid content of the fungal tissue.

Chemical Determinations.

Nitrogen was determined by the micro-kjeldahl digestion method of Minari & Zilverschmidt (1963). The ammonium sulphate formed was estimated using the Nessler reagent of Koch & McMeakin (1924).

Phosphorus analyses were performed using the ammonium molybdate method of Chen et al (1956).

Carbohydrate was estimated as glucose equivalents using the phenol-sulphuric acid method of Dubois et al (1958).

RNA and DNA were determined by the orcinol and diphenylamine methods respectively, described by Schneider (1957).

Each value obtained was the average of triplicate samples taken from at least four extraction experiments.

(h) Estimation of Riboflavin.

(i) Mycelium

The total amount of riboflavin in the mycelium was measured using the method of Strong (1955). 200mg dry weight of cells were extracted in 20ml 0.1N hydrochloric acid at 121° for 30min, cooled in the dark, the extract adjusted to pH 6.0 then immediately taken to pH 4.5, and the volume of the extract made up to 50ml. 4 x 10ml samples were pipetted into boiling tubes and 1ml water added to one pair of duplicates, to the others were added 1 ml riboflavin solution (1 ug). The extracts were then treated successively with 1ml glacial acetic acid, 0.5ml 3% potassium permanganate followed after exactly 2min with 0.5ml 3% hydrogen peroxide. Gas bubbles were allowed to disperse before samples were read on an Aminco Spectro-photofluorometer, consisting of two monochromators, one serving as a source of exciting light, the other for the fluorescence. To remove interfering fluorescing material, after the samples were read, 20mg sodium dithionite was added to each tube, and the samples again read within 30 seconds.

(ii) Culture filtrates.

Culture filtrates were adjusted to pH 4.5 and samples then treated as above. Care was taken to ensure that all practical manipulations in these determinations were carried out under dim light.

(iii) Chromatography of Culture Filtrates.

Preliminary experiments were undertaken to find the most suitable method for extraction of culture filtrates to obtain samples suitable for chromatography. Freeze dried culture filtrates were reconstituted in 1/10th of their original volume of distilled water and extracted twice with 2ml of a saturated phenol solution (Yagi, 1962). The combined phenol extracts were then in turn extracted with diethyl ether and water, centrifuged and the aqueous phase removed for chromatography. Although this method gave satisfactory results, better subsequent chromatographic separation was obtained when culture filtrates were purified by the method of McNutt (1954), as follows:- Concentrated culture filtrates were brought to pH 5.5 and the solutions passed through a column of magnesium trisilicate (Florisil, Sigma Ltd.,), of dimensions 1cm x 20cm. This was then washed with 500ml water. The column was sucked dry and the yellow band adsorbed at the top extracted in the dark four times with pyridine + acetic acid + water (6ml + 0.6ml + 23.4ml). The combined extracts were taken to 2ml on a Rotary evaporator, made up to 5ml with pyridine, 200ml of acetone then added, and the precipitate which formed was removed by filtration. The concentration and acetone precipitation was repeated twice more, and the volume of the

extract finally taken up in 2ml pyridine. This procedure seemed to remove most of the interfering material with very little loss of riboflavin. The riboflavin was then further purified by descending one way paper chromatography on Whatman 3mm paper using the butan-1-ol + acetic acid + water (4 + 1 + 5 v/v) solvent system described by Kilgour, Felton & Huennekens (1957). Detection was by direct vision or by viewing under a U.V. lamp (Camag TL 900) at 254nm. This procedure gave much better separations than those using silica gel G and Whatman No.1 paper with the same colvent system.

(i) Estimation of Keto Acids.

Pyruvate and ∝oxoglutarate were estimated by the method of Kanazawa et al (1967). Cells were extracted with 1.5N perchloric acid at 0° twice for 30min, washed with 1N perchlorate, the extracts combined and the perchlorate removed by titration with 10% potassium hydroxide to pH 6.8. After incubation with 0.2% dinitrophenyl hydrazine in 2N hydrochloric acid, the DNP derivatives of the keto acids were extracted three times with redistilled ethyl acetate which were in turn extracted with 10% sodium carbonate solution. The absorbance of these solutions was then measured at 355nm and 400nm, and the amounts of pyruvate and ∝oxoglutarate calculated using the equations:-

$$\text{Pyruvate (umole/ml)} = (1.22\ E_{355} - E_{400})/6.6$$

$$\text{∝Oxoglutarate (umole/ml)} = E_{400} - 0.76E_{355})/6.6$$

Keto acid concent of culture filtrates were estimated by acidifying the samples (5ml) with 15ml 10% TCA., centrifuging to remove protein, and 3ml of protein free solution used for analysis as described above.

(j) Extraction and Separation of Lipids.

Lipids were extracted according to the method of Bligh & Dyer (1959). 300mg dry weight of cells was extracted with 30ml methanol + chloroform (v/v 1 + 1) in a Potter Elveheim homogeniser at 0°. A further 10ml chloroform was added, and after further homogenisation for 1 min, finally 10ml water. The total was centrifuged and the chloroform layer separated and dried over anhydrous sodium sulphate. For chromatography, the final volume of the extract was concentrated to 0.5 ml by evaporation under reduced pressure at room temperature.

Lipids were separated by thin layer chromatography on 20 x 20cm plates of Silica Gel G using the solvent system suggested by Marinetti et al (1959) consisting of di-isobutyl ketone + acetic acid + water (80 + 50 + 7 v/v). The samples, 20 1, were applied and dried under nitrogen, and the chromatograms run in the dark in tanks lined with a filter paper wick which ensured conditions of solvent saturation of the tank.

Separated components were detected by spraying the plates with a methanolic solution of phosphomolybdic acid followed by gentle heating when the components appeared as blue spots on a faint yellow background. Phospholipids were detected using a molybdate - perchlorate spray which gave immediate appearance of blue spots on a white background.

(k) Estimation of Sterol Content.

Sterols were extracted from the fungus by the method described by Klieber, Payne & Appleton (1955). Cells (200mg dry weight) were

hydrolysed for 1h at 121° in 20ml 5N sodium hydroxide and the sterols removed with diethyl ether. This organic extract was dried over anhydrous sodium sulphate, evaporated to dryness under reduced pressure and finally redissolved in chloroform and made to volume. Quantitative estimation was performed by the Liebermann Burchard colorimetric method as modified by Stoudt & Foster (1954). Values were obtained from a standard curve prepared by treating known quantities of ergosterol (0-250µg) in the same manner as the samples.

(l) Glucose uptake by Cells.

Cells were grown in 250ml conical flasks at 25° in 25ml of the chemically defined medium described previously, containing 2% w/v glucose and 0.1% w/v glycine. These concentrations were employed since they gave a suitable measurable range of residual carbon source under the conditions of the experiments.

Glucose was estimated using the Blood Sugar test combination of Boehringer & Co Ltd., employing glucose oxidase, peroxidase and O-dianisidine. Cells were filtered, washed three times with distilled water and the filtrates assayed. Results were expressed as mg glucose consumed per g dry weight of fungus.

(m) Pyrolysis of cells for Fingerprinting by G.L.C.

Pyrolysis products from cells were measured by pyrolysis followed by gas liquid chromatography as described by Myers & Watson (1969). A Pye series 104 Model 24 gas liquid chromatograph was used with dual flame

ionisation detector, pyrolysis assembly and Pyrex glass columns 1.5m long x 4mm diameter, packed with 'Carbowax 20M' plus phosphoric acid (solvent dichloromethane). Comparative freeze dried samples were taken from cells grown in light and dark, inserted in the pyrolysing element and pyrolysed at 800°. Analyses were carried out in a stream of nitrogen (45ml/min) under a programmed rise in temperature of 8°/min from 40° at the start of the run to 140°.

(n) Determination of Catalase Activity.

Catalase activity was measured iodometrically (Luck, 1965). 10ml cell free extract in $^{M}/_{150}$ phosphate buffer pH 6.8, or 10ml culture filtrate was added to a flask containing 90ml 1% hydrogen peroxide in $^{M}/_{15}$ phosphate buffer, pH 6.8 at 0°. After 0.5, 1, 2, 4, 7, 10 and 15 min, 10ml samples of this suspension were withdrawn and the residual H_2O_2 assayed iodometrically by titration against 0.05N sodium thiosulphate. The velocity constant was determined for each analysis according to the equation $K = \frac{2.3}{t} \log_{10} \frac{\text{Initial } (H_2O_2)}{\text{Residual } (H_2O_2)}$

The value for k_o was obtained by extrapolation to t_o and corrected for mg protein of sample, as a measure of activity.

(o) Protein Estimations.

Protein was estimated by the method of Lowry et al (1951) involving the reaction of phenol groups in tyrosine and tryptophan with the Folin reagent to give a blue colour. Values were obtained by reference to a standard line using bovine serum albumin in concentrations of 10-280µg/ml).

Methods used in Carotenoid Production by Cephalosporium diospyri

(i) Production of Carotenoids.

To obtain sufficient quantities of carotenoids for detailed chromatographic analyses, the organism was grown for seven days in the medium of Schopfer (1934) under continuous illumination at 25°. Following incubation, the cells were harvested by centrifugation and freeze dried.

(ii) Extraction and Separation of Carotenoids.

The method described by Davies (1965) was used. Carotenoids were extracted from the fungus by macerating with acetone in a Waring Blender, filtering through a sintered glass funnel and re-extracting the residue until the filtrate was colourless. The acetone extract was then mixed with half its volume of diethyl ether and water added to make the solution diphasic. After the aqueous phase had been extracted to completion with ether, the ether solutions were combined, washed with water, dried over anhydrous sodium sulphate, removed by filtration and finally taken to dryness under reduced pressure on a rotary evaporator.

This lipid residue was saponified by redissolving it in absolute alcohol, and then 60% (w/v) aqueous potassium hydroxide added. The alkaline mixture was left in the dark at room temperature under nitrogen overnight, diluted with three volumes of water and the carotenoids in the upper layer extracted to completion with diethyl ether. These ether extracts were combined, washed free of alkali with water until no longer alkaline to phenolphthalein, dried by standing over anhydrous sodium sulphate, and taken to dryness under reduced pressure. The carotenoids

were stored in the dark under nitrogen.

Sterols were removed by dissolving the unsaponifiable fraction in light petroleum which was then stored overnight at -20°. The sterols which had precipitated were removed by filtration and the filtrate again taken to dryness and stored under nitrogen.

The dried residue was taken up in 2ml hexane and chromatographed on a magnesium oxide + celite (1 + 1) column. Development was by washing with increasing concentrations of ether in hexane. The bands were eluted, taken to dryness redissolved in light petroleum, and their absorption spectra estimated on a Unicam Series II SP 500.

Carotenoids were also separated by thin layer chromatography on 20 x 20cm. plates of Silica Gel (Merck) at a thickness of 0.3mm. in various solvent systems as described later, and detected either visibly or by exposure of plates to iodine vapour (Davies et al, 1961), as well as their absorption spectra after elution.

(iii) Experiments on Factors affecting Carotenogenesis.

Fungi were grown on Schopfer's medium for seven days, harvested and washed three times with 50ml M/60 phosphate buffer, pH 5.6, containing 2% glucose and 0.1% glycine. All procedures were carried out aseptically under light from a Red Safe light (Kodak). Finally the fungi were suspended in 25ml amounts of the buffer in 250 ml conical flasks. Following incubation, the fungal suspensions were transferred to centrifuge tubes and 0.5ml 40% (w/v) TCA added. After centrifugation the supernatant was discarded and the cells washed and freeze dried. These cells were then extracted and their carotenoid content measured.

For the purposes of this investigation, it was decided, in view of the number of samples to be assayed, to find a method of extraction which was quick, simple and reproducible. After preliminary trials, a procedure was adopted which was suitable. Cells (50mg dry weight) were extracted in three times 5ml acetone + methanol (7 +2, v/v), using a mechanised Potter Elveheim homogeniser driven by a Gallenkamp small electric motor, the extracts combined, transferred to petroleum spirit (b.p. 40-60), and the absorbance of the clear solutions measured at 470nm on a Unicam Series II SP 500. Carotenoid content was calculated assuming a molar coefficient of extinction of 3000. It is emphasised that values obtained are only used comparatively and not absolutely. Results are expressed as μg carotenoids per g dry weight of the fungus. In experiments where the organism was exposed to light, the incubation conditions were identical to those described previously, except where otherwise stated.

Inhibitors when soluble in water were added in aqueous solution. Compounds relatively insoluble in water such as diphenylamine were added in ethanol, never exceeding 0.25ml ethanol per 50ml incubation mixture. All compounds used were sterilised by membrane filtration.

Thin Layer Chromatography.

The apparatus supplied by Shandon and the media of Merck and Co. were used in all the chromatography performed in this work. Saturation chambers (Davies, 1963) were also used for analysis of carotenoid pigments.

Washing of Glassware

Dirty glassware was soaked in a 2% solution of Decon 75 for at least 24h, thoroughly rinsed in tap water, soaked in distilled water and air dried.

Chemicals.

Where possible, all organic and inorganic chemicals used were Analar grade obtained from B.D.H. (Poole). Organic solvents were checked before use for presence of peroxides by the method Moore & Stein (1954).

Parahydroxymercuribenzoate, parahydroxymercuribenzenesulphonic acid and Antimycin A were all obtained from Sigma Ltd., Iodosobenzoic acid was supplied by Koch Light Laboratories Ltd. Mepacrine was kindly supplied as a gift from I.C.I. Pharmaceuticals Ltd., Wilmslow, Cheshire. Parachloromercuribenzoate was purchased from Calbiochem Ltd.

RESULTS.

1. Growth of the organism

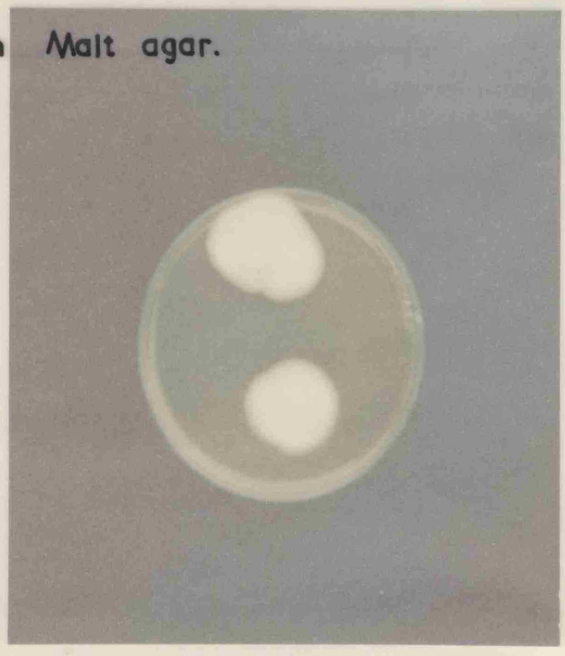
During this investigation, it was noticed when subculturing the organism from soil on to solid media that C.diospyri was able to grow as two distinct morphological forms: the normal type and a 'yeast' like form. (Plate 1). These 'yeast' forms grew as small single colonies consisting of masses of clumped single cells producing germ tubes or pseudomycelia which then appeared to fragment to the single cell type. The factors responsible for this dimorphism have not been discovered, but it would appear that there is little similarity between this instance and other reported cases of dimorphism in fungi (see Romano 1966 for review), where temperature and nutrition were the controlling factors in the mycelium - yeast transformation. This 'yeast' form appeared to be reasonably stable over at least ten subculturings on to malt agar plates, but at the peripharies of the colonies were produced white sticky clusters of aerial hyphae, due possibly to a partial reversion to the mycelial form when able to grow free from the film of yeast like cells on the solid medium.

It was also noticed (Plate 1) that when grown on Malt agar, this 'yeast' form produced pigments even when grown in complete darkness. They were carotenoid in nature, as expected, since an extract in acetone gave a blue colouration with conc. sulphuric acid (Karrer & Jucker, 1950). Because of this unexpected complication, all inocula were checked for the presence of these light independent forms in the inoculum by allowing the organisms to grow on malt agar for 6 days in the dark at 25^{o} when the appearance of small pink colonies were indicative of their presence. Only inocula free of this yeast form was used in all the experiments reported, and checks were carried out at all stages in the inoculation procedure. These dimorphic forms did not produce pigment in the dark when grown on solid Schopfer's medium or Czapex dox agar, possibly

Plate I

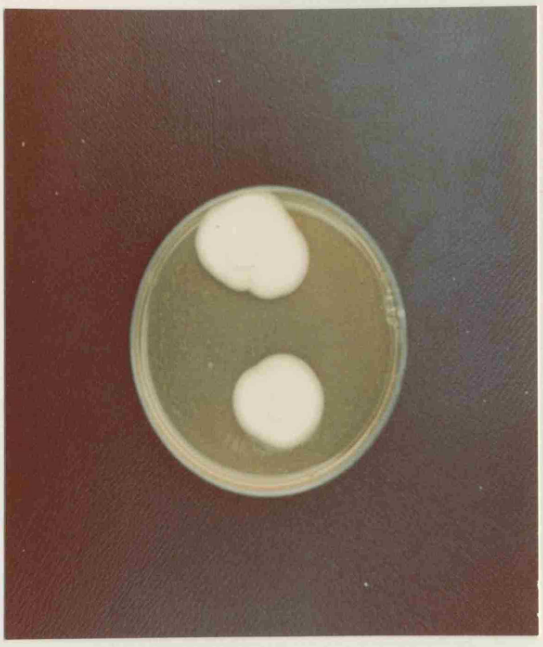
Dimorphism exhibited by C. diospyri.

Cells grown at 25° for seven days in the dark on Malt agar.



A Normal colourless growth.

B Pigmented yeast form.



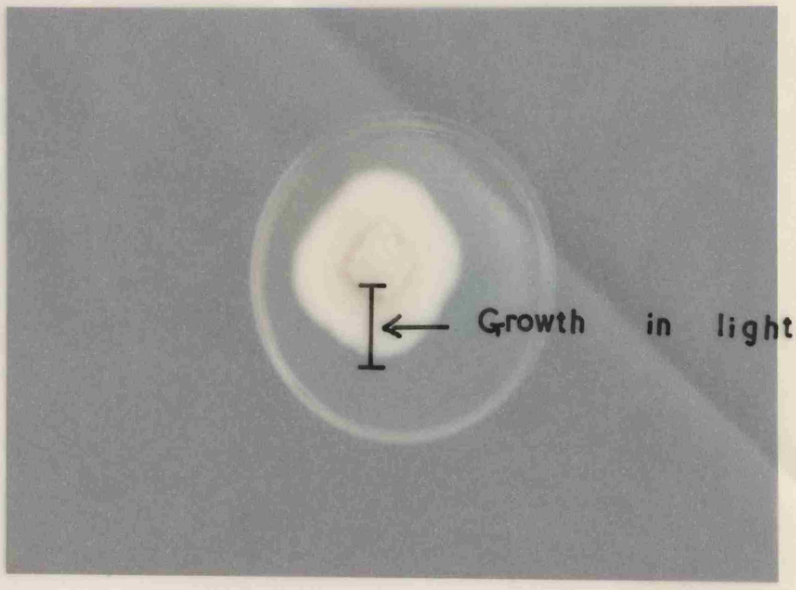
implicating the direct or indirect involvement in pigmentation of nutritional conditions. Attempts to grow cells in stationary liquid cultures in Schopfer's medium were unsuccessful. In both malt broth and Czapek dox broth, spores on inoculation sank to the bottom of the liquid where they germinated and after about three days floating colonies became established, from spores which were presumably trapped in the surface tension layer. These resulted finally in the production of a thick surface pellicle of cells of different physiological age and subject to a differing degree to the nutritional and oxygen tension gradients set up under such conditions of growth. Stationary cultures grown on Schopfer's medium did not form surface pellicles but grew as a film on the bottom of the vessels (20 oz. medical flats containing 50ml medium which gave a large surface area: volume ratio).

In shake cultures, cells grown in both malt broth and Schopfer's medium were not truly mycelial, but mainly in the form of mycelial fragments and large amounts of conidia, whereas those grown in Czapek Dox broth grew as mycelial clumps. This wide difference in growth form, due again possibly to nutritional composition of the media, illustrates the difficulty encountered in comparing the growth of this organism in the three media. For various reasons, the most important of which was the ability to control the chemical composition to a larger extent, Schopfer's medium was chosen for all analytical experiments, and the other two only as comparisons.

Submerged cultures of C.diospyri grown in both malt broth and Schopfer's medium became progressively more viscous during incubation due to production of extracellular material. This high molecular weight material could be precipitated from culture filtrates with two volumes of 95% ethanol, and by analogy with the findings of Buck et al (1968), was most probably a glucan. A freeze dried sample was white which

Plate II

C. diospyri grown on Malt agar at 25°
for four days under continuous light
then replaced in darkness for a
further four days.



although soluble in the original culture medium was sparingly soluble in distilled water and fresh Schopfer's medium. Light and dark grown cultures of C.diospyri produced this polymer material. Cells grown on Czapek dox broth however, were found not to form this ethanol insoluble material up to and after growth had ceased, an observation which would appear to agree with the findings of Buck et al (1968) who, with Claviceps fusiformis noticed that potassium nitrate out of a wide range of nitrogen sources tested did not support glucan production by that organism.

2. Pigment Production by C.diospyri.

Light grown cultures of C.diospyri on a wide range of both solid and liquid media produced pink coloured cultures, whilst those grown in complete darkness remained colourless. Centrally inoculated plates of malt agar were grown at 25^{o} under 2000 ft candles for 6 days to produce strongly pigmented colonies. When replaced in the dark, all further growth resulted in non-pigmented hyphae (Plate II). In addition, there appeared to be no morphological changes such as zonation or lack of reproductive structures induced by the removal of the light stimulus. Similar results were obtained when dark grown colonies on malt agar plates were partially illuminated with a parallel beam of light. There was no carotenogenesis in mycelium adjacent to but not within the zone of illumination. These results would suggest that the influence of light in inducing carotenogenesis could not be transmitted from illuminated sections of the mycelium to adjacent mycelium in darkness.

Effect of Medium on Carotenogenesis.

Table II shows the carotenoid content of cells of C.diospyri grown in submerged culture for 6 days under continuous illumination in different media. It would appear that cells grown in malt broth contained more pigment than those grown in Schopfer's medium, and these, in turn, contained more than those grown in M/60 phosphate buffer, pH 5.8 containing

2% glucose and 0.1% glycine (afterwards referred to as phosphate medium). Therefore nutritional conditions were extremely important in determining the carotenoid levels in C.diospyri, and the most important limiting factor in their synthesis could well be the absolute levels and the C/N ratio of the medium in which the cells are grown.

TABLE II.

Carotenoid content of C.diospyri grown under continuous illumination at 25° for 6 days. Mean of triplicate samples, two experiments.

Medium used.	Carotenoid content of cells µg/gm dry weight of fungus	
	(a)	(b)
Malt broth	514	502
Schopfer's Medium	238	254
Phosphate Medium	118	131

Composition of the Carotenoids of C.diospyri.

This exercise was undertaken as a preliminary investigation and, for this reason, only the epiphasic pigments of light and dark grown cells were analysed and the carotenoid fractions tentatively identified.

Light Grown Cells.

The unsaponifiable fraction run on a 10cm x 1cm column of MgO + Celite (1 + 1 w/w) and developed with 100ml hexane followed by 100ml 4% (v/v) ether in hexane gave the chromatogram described below. The zones are numbered in order of decreasing adsorptive power.

Zone I: orange red pigment, very strongly adsorbed at the top of the column which did not run even in absolute ethanol.

Zone II: bright pink-red pigment - major zone.

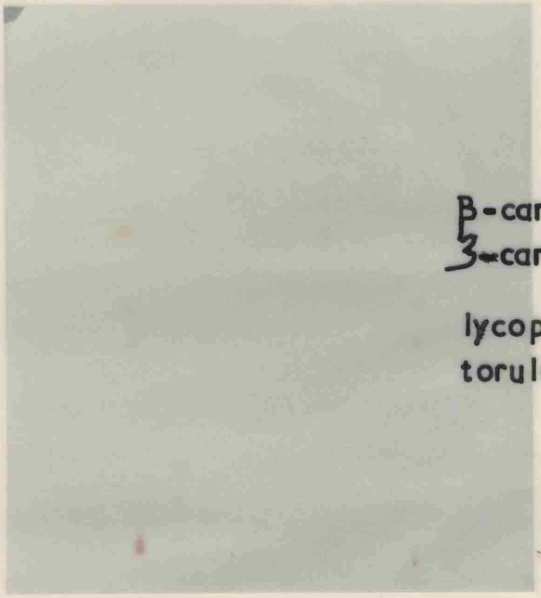
Zone III: orange pigment.

Zone IV: pale yellow pigment

Zone V: orangy-yellow pigment

Plate III

Separation of epiphasic carotenoids of C. diospyri grown in Schopfer's medium in the light on Silica Gel G. Developing solvent was Light Petroleum + Methylene Chloride (8 + 2 $^{v}/_{v}$)



I II III

I - Total Extract.

II - β-carotene purified by chromatography on MgO + Kieselguhr G (1 + 1 $^{w}/_{w}$) in the solvent system hexane + acetone (9 + 1 $^{v}/_{v}$).

III - The remainder rechromatogrammed after separation in the same manner as described for II.

The same pattern was obtained when the carotenoids were separated on a column of the same dimensions containing Alumina (Brockmann activity Grade I) and developed with 4% (v/v) acetone in light petroleum (b.p. 40-60).

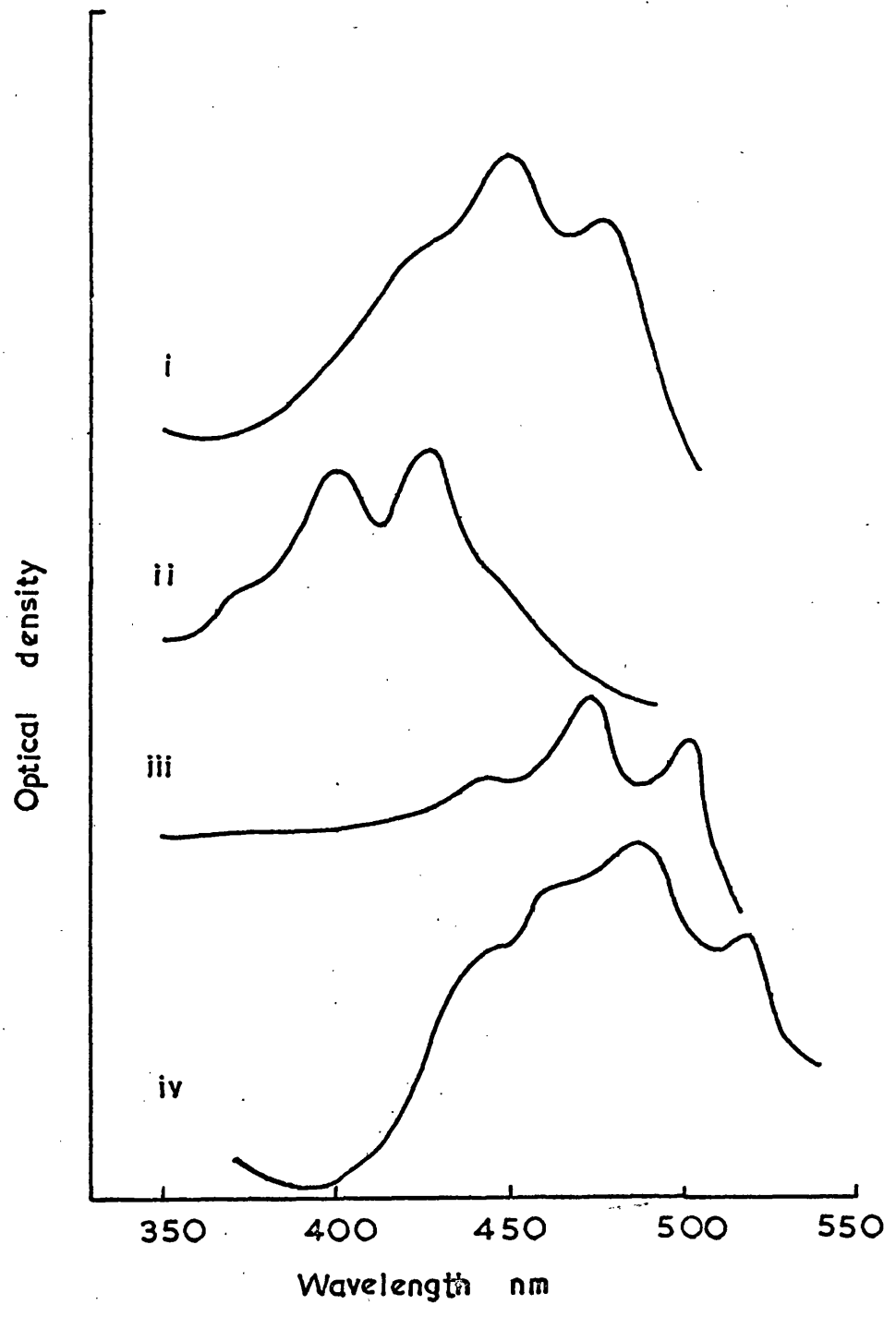
Thin layer chromatography was also used as an attempt to separate the epiphasic carotenoids. All plates were run in saturation chambers. Pigments run on plates of silica gel G in the solvent system light petroleum + benzene (1 + 1 v/v) were resolved into three well defined components; (i) a yellow spot running with the solvent front, (ii) a red spot with an Rf of about 0.65, (iii) a red band just off the origin. As well as these, and in all the separation techniques, there was a red non-moving component at the origin. Similarly, pigments separated in the same solvent system on silica gel G + calcium hydroxide (1 + 4 w/w) gave reasonable separation with (i) the yellow spot running with the solvent front, (ii) an orange red spot well defined and running just behind the yellow component, (iii) an orange band which had moved just off the origin. The most satisfactory separation was obtained with silica gel G as the adsorbant and light petroleum + methylene dichloride (80 + 20 v/v) as the developer. A typical separation is shown in Plate III. It can be seen that the epiphasic pigments had separated into four well-defined spots. However they appeared to be very unstable in this solvent system on drying and faded completely in less than 1 min. Because of this no identification could be made from this separation.

Carotenoids chromatogrammed on plates of MgO + Kieselguhr G (1 + 1 w/w) in the solvent system hexane + acetone (90 + 10 v/v) also gave good separation of components and their colour appeared to be stable on dry plates. Again, four well defined spots were noticed; (i) an orange yellow spot with an Rf of approx 0.8, (ii) a pale yellow spot with an Rf of approx. 0.65, (iii) a red spot with an Rf of approx. 0.55, and (iv) a red band with an Rf of approx. 0.15. In addition, the bright red

FIG.2.

Absorption spectra of epiphasic pigments of cells of C.diospyri grown under continuous illumination in Schopfer's medium at 25°. Pigments were initially separated by thin layer chromatography as described in the text. Readings were performed in light petroleum (b.p. 40-60).

i) β-carotene
ii) ζ-carotene
iii) lycopene
iv) torulene


Optical density
i
ii
iii
iv
350
400
450
500
550
Wavelength nm

non-moving spot on the origin was noticed. These four separated compounds were eluted from the adsorbent with 3 x 5ml amounts of hexane + ethanol (4 + 1 v/v), taken to dryness, and their absorption spectra read in light petroleum (b.p. 40-60). Spectra obtained are shown in Fig. II. From these the epiphasic pigments of C.diospyri were tentatively identified as (i) β-carotene, (ii) ζ-carotene, (iii) lycopene, (iv) possibly torulene. No effort was made to identify other pigments remaining at the origin on the chromatograms. Although no authentic samples of these compounds were obtained, the absorption maxima of the carotenoids from C.diospyri were in good agreement with published values for these pigments. (Table III). This method of separation gave the four pigments corresponding to those obtained with the solvent system light petroleum + methylene dichloride (4 + 1 v/v) as can be seen in Plate III.

TABLE III

Comparison with Literature values of the Absorption maxima of carotenoid pigments isolated from C.diospyri. Values taken from Davies, (1965).

	Wavelength maxima (nm)					
Compound	Experimental			Literature		
Zeta-carotene	372	400	423	376	398	418
β-carotene	425	451	475	423	450	480
Lycopene	445	472	502	445	472	504
Torulene	462	486	514	460	484	518

In the case of torulene, there was obvious interference with the appearance of a small peak around 445nm, due possibly to contamination with another pigment. Attempts to further purify this compound on Silica Gel G plates was unsuccessful, since in all the solvent systems described, only a single component was obtained.

Colourless Precursors and Carotenoids of dark grown cells.

The unsaponifiable epiphasic pigments in light petroleum from both light and dark grown cells of C.diospyri were chromatogrammed on columns of alumina (Brockmann activity Grade I), and the fractions collected in the case of the extract from light grown cells before the first coloured band was eluted. This was usually achieved with 100ml light petroleum followed by 50ml 2% ether in light petroleum. The same volume was collected when pigment from dark grown cells was separated. In neither case was any ultra violet fluorescing material noticed moving down the columns. Dark grown cell extracts, however, after development did show a band of fluorescing material adhering to the top of the column, due possibly to unprecipitated sterol.

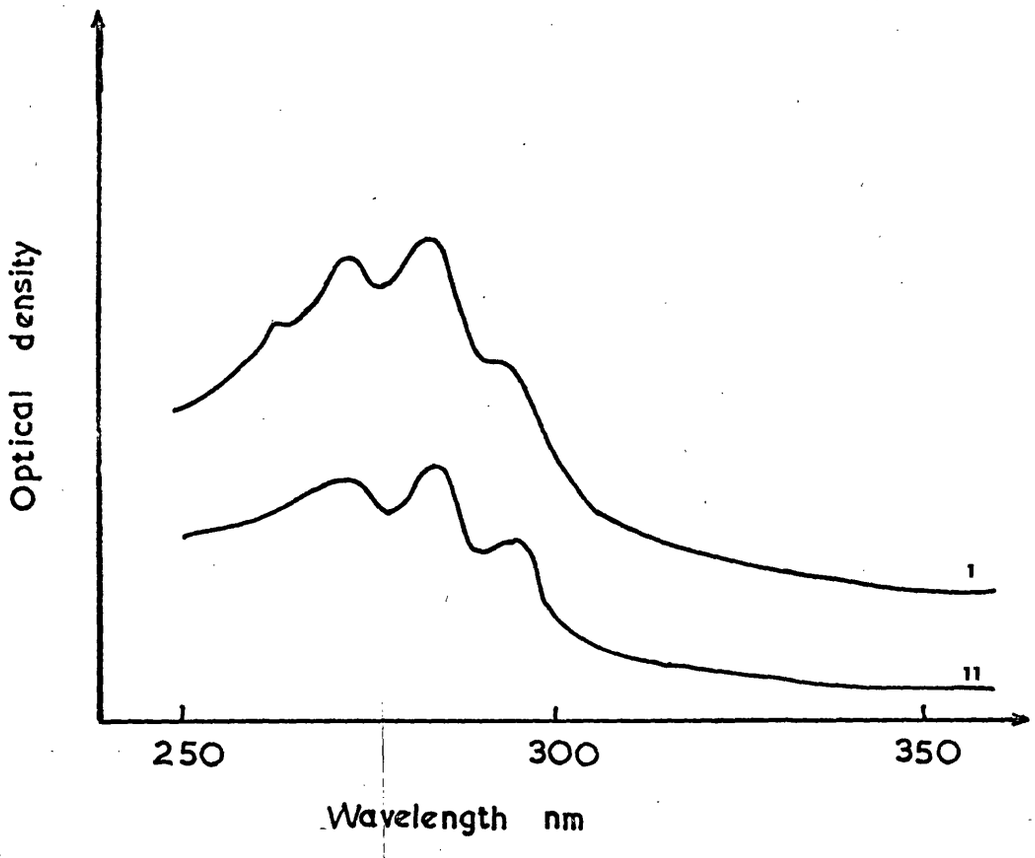
Absorption spectra of these first fractions in light petroleum (b.p. 40-60) showed the presence of large amounts of interfering u.v. absorbing material, since there was a gradual decrease in optical density from OO at 250nm to 0.1 at 370nm with no indication of any maxima. When re-chromatographed on silica gel G and developed with light petroleum, and the plates exposed to iodine vapour (obtained by dropping a few crystals of iodine onto hot sand in a crucible) both samples showed the presence of a large amount of interfering material at the origin as well as a single spot with an Rf of approx. 0.2. This value compares well with that quoted by Mercer et al (1963) for phytoene. No component was noticed which might have conceivably been due to phytofluene, and together with the lack of fluorescence material on developed column chromatographs, suggests the absence of this precursor in any detectable amounts in both light and dark grown cells of C.diospyri.

Chromatography of the fractions from column chromatographs on plates of MgO + Kieselguhr G (1 + 1 w/w) allowed separation of the precursors, and elution from the adsorbant with 10% ethanol in hexane, gave absorption spectra as shown in Fig III. The spectra obtained possessed the characteristic curve for phytoene with maxima at 273, 284 and 294 nm, values which

FIG.3.

Absorption spectra of colourless precursors of carotenoid pigments of C.diospyri grown in Schopfer's medium for 6 days at 25°. Separation of pigments as described in the text. Spectra were read in light petroleum (b.p. 40-60).

i) Phytoene from light grown cultures
ii) Phytoene from dark grown cultures.


Optical density
1
11
250
300
350
Wavelength nm

compare well with the published maxima (Davies, 1965), for authentic phytoene. The extra small peak present in the light grown extract was probably due to interfering material at 263nm. No other carotenoid pigment was detected in dark grown cells of C.diospyri. From this evidence it might appear that the block in carotenoid synthesis in dark grown cells occurs after the synthesis of phytoene, and that light in some manner allows the expression of an enzyme capable of forming the more unsaturated coloured pigments. Failure to detect phytofluene in light grown cells might suggest its presence in very small amounts as an intermediate due to rapid turnover. On the other hand, the absence of phytofluene in dark grown cells would imply that phytoene was the end point.

(3). Mechanism for Photoinduction of Pigments.

C.diospyri, like other fungi, had an absolute requirement for light in carotenoid production, and also like some other fungi was able, on subsequent incubation in the dark after exposure to light, to synthesis their pigments. As already mentioned, only cells illuminated and not their dark grown progeny behaved in this manner, and for this reason to obtain suitable conditions for experimentation, non-proliferating populations of cells were incubated in M/60 phosphate buffer, pH 5.8. It was found necessary to supply glucose and glycine for the cells to carry out carotenogenesis. The response to various glucose concentrations is shown in Table IV. It can be seen that carotenoid production was approximately the same at all glucose levels above 1%. For all experiments therefore, 2% glucose was incorporated into the buffer medium. Glycine (0.1% w/v) was also added.

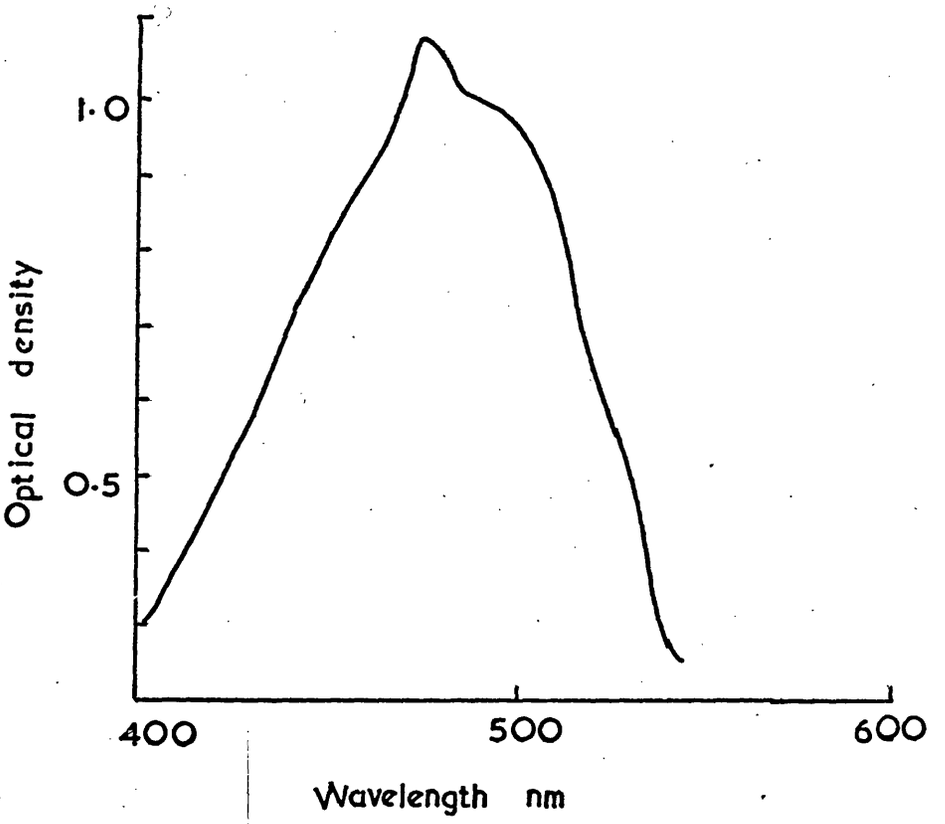
TABLE IV.

Effect of glucose concentration on cells of C.diospyri incubated in M/60 phosphate buffer pH 5.8 containing 0.1% glycine cells were incubated under 2000ft candles of illumination at 25^{o} for 6 days.

Glucose Concentration % w/v	0	0.5	1.0	2.0	3.0	4.0	5.0
Carotenoid content µg/g dry weight	—	92.8	109.6	114.6	110.2	115.6	113.6

FIG.4.

Absorption spectrum of the crude acetone + methanol (7 + 2 v/v) extract of cells grown under illumination for 6 days at 25^{o} in phosphate medium. Pigment transferred to light petroleum spirit (b.p. 40-60) for reading.


1·0
0·5
Optical density
400
500
600
Wavelength nm

Absorption Spectrum of Crude Carotenoid Extract.

A crude 7 + 2 (v/v) acetone + methanol extract of freeze dried cells of C. diospyri grown in Phosphate medium was taken to dryness, re-dissolved in light petroleum (b.p. 40-60) and its absorption spectrum measured as shown in Fig IV. The main peak was located at 475nm and for all quantitative measurements, samples were read at this wavelength and carotenoid content estimated using a $E^{1\%}_{1cm}$ of 3000.

Time course for Carotenogenesis.

Cells incubated under continuous illumination in phosphate medium produced carotenoid as a function of time as shown in Fig V. There was a lag period of about 24h in the light before carotenoid synthesis started followed by a linear increase up to 48h after cessation of increase in biomass. For future experimental work therefore, it was decided to incubate primarily for three days in the dark to allow any growth which did occur to cease, and then expose the fungi to light. This method gave the results shown in Fig VI. In this instance, the lag period before onset of detectable carotenoid formation was only approx 6h with a linear increase up to 72h.

Since C.diospyri had no requirement for continuous illumination, further studies on the initial photoinduction were carried out. Cells were exposed to light for various lengths of time, further incubated in the dark for 72h after which time their carotenoid content was estimated. Results are shown in Fig. VII. This organism therefore required at least 30 min light exposure and periods of illumination above 90 min were of no greater effect. Similar results were obtained when 6 day old cultures grown in the dark on Malt agar plates were exposed to light. Under conditions of continuous illumination, there was a much larger production of pigment than in cells initially illuminated and subsequently dark incubated (approx. 2x). One theory to account for these results suggests that in dark grown organisms,

FIG.5.

Time curve for carotenogenesis of cells of C.diospyri incubated in phosphate medium under continuous illumination at 25°. Carotenoids were estimated as described in the Materials and Methods section.

●—● dry weight production

○—○ carotenoid content.

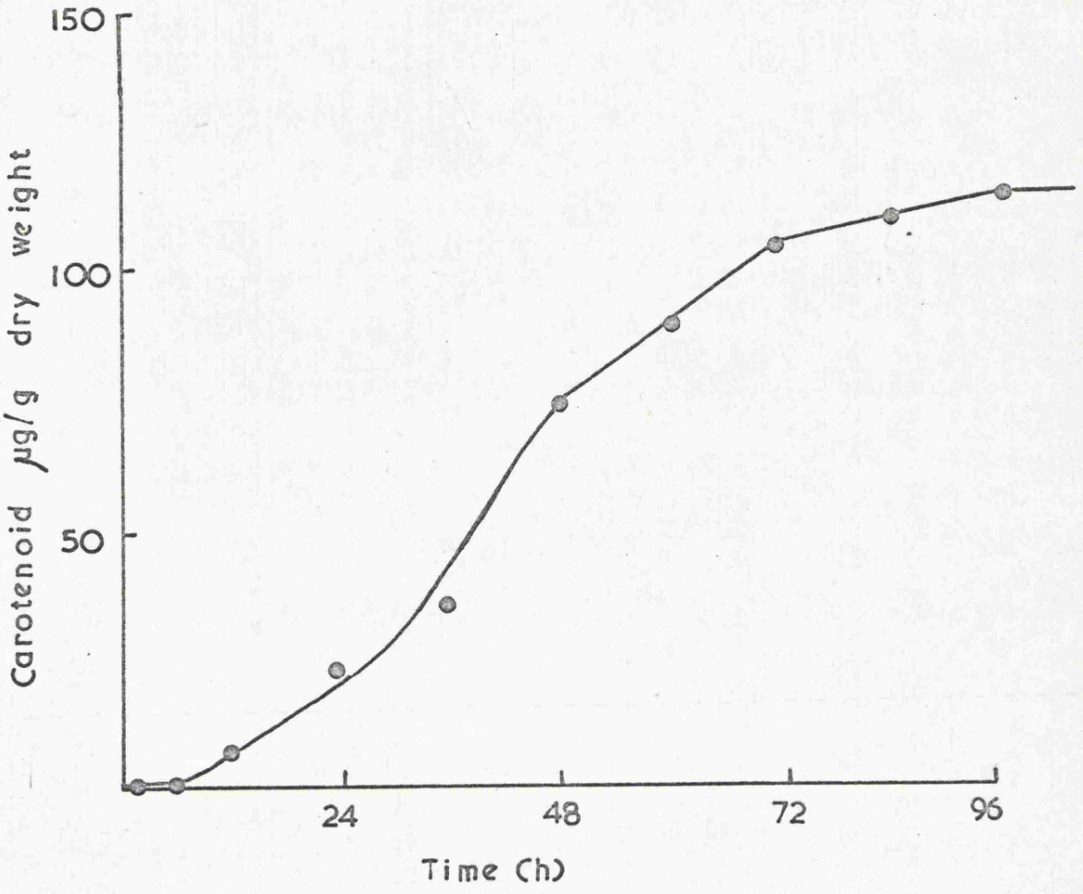
Dry weight in mg
100
200
Carotenoid content µg/g dry weight
50
100
24
48
72
96
120
144
168
Time (h)

FIG.6.

Time course for appearance of carotenoids in cells incubated for three days in the dark, then exposed to light. Phosphate medium was used at 25^{o} and the carotenoids estimated as described previously.

FIG. 7.

Dependence of carotenogenesis on the duration of light exposure in C.diospyri. Cells were incubated for three days in phosphate medium, exposed to light for the various times, replaced in the dark and their carotenoid content estimated after three days.


150
100
50
Carotenoid µg/g dry weight
24
48
72
96
Time (h)

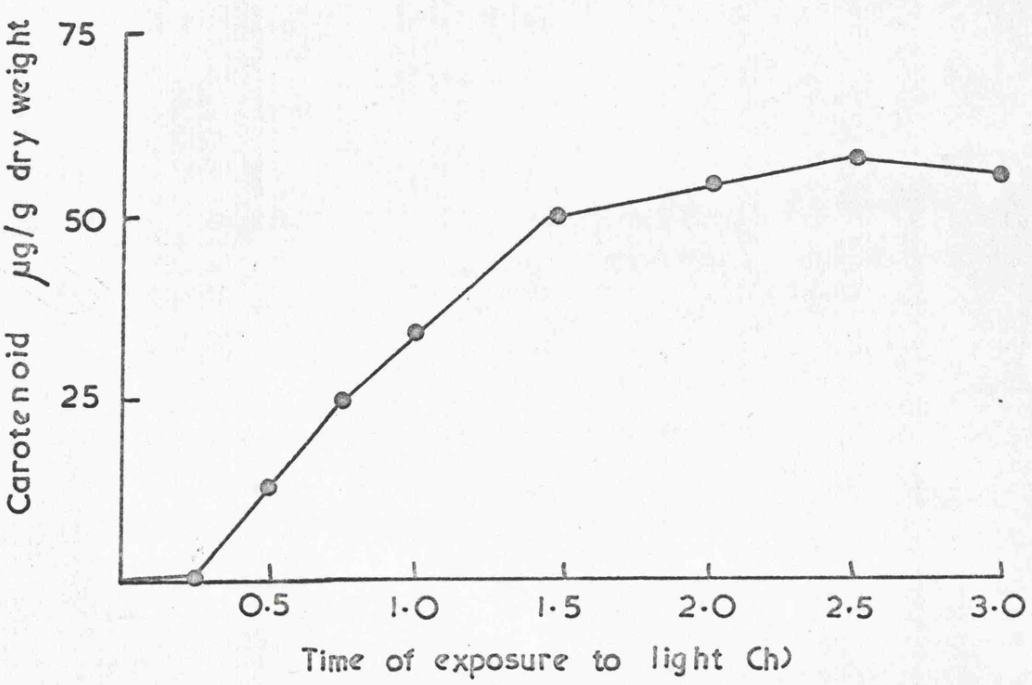
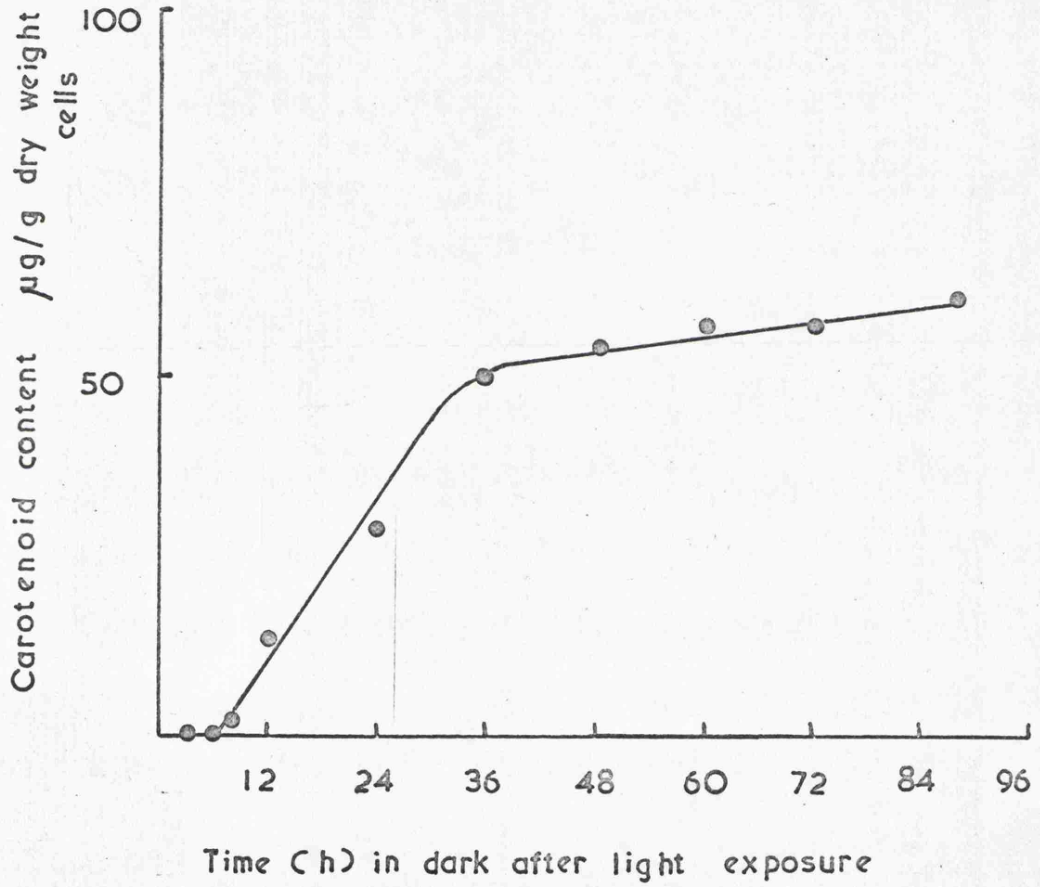

75
50
25
Carotenoid µg/g dry weight
0·5
1·0
1·5
2·0
2·5
3·0
Time of exposure to light (h)

FIG.8.

Time dependence of carotenogenesis in cells of C.diospyri incubated in phosphate medium for three days in the dark, exposed to light for 90 min, then replaced in the dark.


Carotenoid content µg/g dry weight cells
100
50
12
24
36
48
60
72
84
96
Time (h) in dark after light exposure

carotenogenesis was repressed. On illumination, there was a process of derepression only for cells to become eventually repressed again during the dark incubation.

Time dependence for carotenogenesis in organisms exposed to light for 90 min and replaced in darkness is shown in Fig. VIII. Again the lag period under these conditions was about 6hr before onset of pigmentation and their synthesis continued for approx 36 hr, less than under conditions of constant light. These experiments suggested that the light dependent production of carotenoids followed closely the process described to occur in N.crassa (Zalokar, 1954), except that this organism appeared to be far less sensitive to light than N.crassa. Further study was undertaken on the mechanism for this photoinduction.

Requirements for Photoinduction of Carotenoids.

Oxygen was found to be absolutely necessary for both the initial photoinduction and the subsequent synthesis of carotenoid pigments. Cells incubated for three days in the dark in the phosphate medium were flushed with white spot nitrogen for 15 min under aseptic conditions, the conical flasks sealed with rubber bungs and the fungi then exposed to light for 90 min. After replacement in the dark for a further 72h, their carotenoid content was measured. Results obtained are shown in Table V.

TABLE V.

Requirement for oxygen in carotenoid synthesis by C.diospyri

Technique was that described in the text.

Conditions of		Carotenoids
Light induction	Dark Induction	µg/g.dry weight
N_2	N_2	negligible
Air	Air	56.8
N_2	Air	negligible
Air	N_2	negligible

From these results it can be deduced that the primary effect of light is most probably a photochemical oxidation, and as such should be

independent of temperature. The data presented in Table VI illustrates this.

TABLE VI

Effect of Temperature on Carotenogenesis in C.diospyri.

Cells incubated in 25 ml phosphate medium for three days in 250ml conical flasks in the dark, and exposed to light under various conditions for 90min. subsequent incubation was in the dark for a further three days. Cells exposed below 0° were frozen as a film in the vessel and exposed under stationary conditions of 2000ft candles.

Induction Temp ($^{\circ}$C).	Incubation Temp. ($^{\circ}$C)	Carotenoids µg/g.dry weight
25	25	53.0
25	-20	negligible
-20	-20	negligible
-20	25	45.5

It would therefore seem that the initial photoinduction was temperature independent, whereas the overall production of pigments was not. Once exposed to light, the fungus was able to produce carotenoids after prolonged delay at low temperature. The results in Table VII indicate that there was little or no decrease in the extent of photoinduction in C.diospyri after 8 weeks storage at -20°.

TABLE VII

Memory of light exposure. Cells incubated for 3 days in the dark, exposed to light for 90 min, then stored at -20° for various times and finally incubated in the dark for a further 3 days in phosphate medium.

Time at -20° (days)	Carotenoids µg/g.dry weight
7	51.5
14	49.4
28	53.8
56	54.2

Determination of approximate action spectrum.

Although results in Table VIII only permit approximate localisation of the wavelengths of light required for photoinduction of carotenoids, it is apparent that only light of a wavelength shorter than 470nm was effective for the photoresponse.

Ilford Colour Filter No.	Wavelength of transmitted light	Carotenoid production
621	380-470	present
622	440-496	present
623	470-520	absent
624	500-540	absent
625	530-570	absent
626	560-610	absent
607	575 upwards	absent

No facilities were available for detailed spectroscopic examination, but the quality of light required for carotenogenesis in this organism seems to fit the general pattern for many photoresponses, especially carotenoid production found in fungi where wavelengths at the blue end of the spectrum are involved.

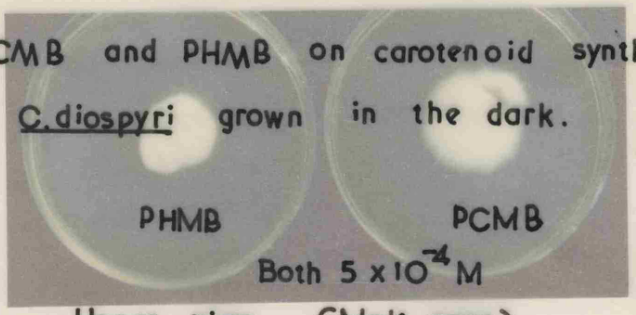
(4). Photomimetic compounds and carotenogenesis in C.diospyri.

PCMB and PHMB known to substitute for light in carotenoid production in F.aquaeductuum (Rau, 1967), were tested for similar properties with C.diospyri. Colonies on Malt agar plates containing PCMB and PHMB were grown at 25^{o} in the dark. The results may be seen in Plate IV. The time lapse before the appearance of pigment was usually about 3-4 days under these conditions of growth, and carotenoids were produced in response to PCMB and PHMB in the concentration range of 5×10^{-4}M to 5×10^{-5}M, higher than those effective in F.aquaeductuum. The result was confirmed using Schopfer's medium + 2% agar (Plate IV).

It may be seen that, like the light dependent pigmentation, only those parts of the fungus in contact with the inducer produced carotenoids. Therefore the immediate alteration in metabolism brought about by these

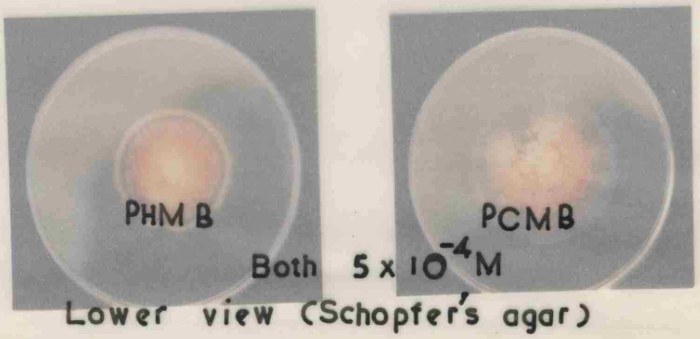
Plate IV

Effect of PCMB and PHMB on carotenoid synthesis on cells of C.diospyri grown in the dark.

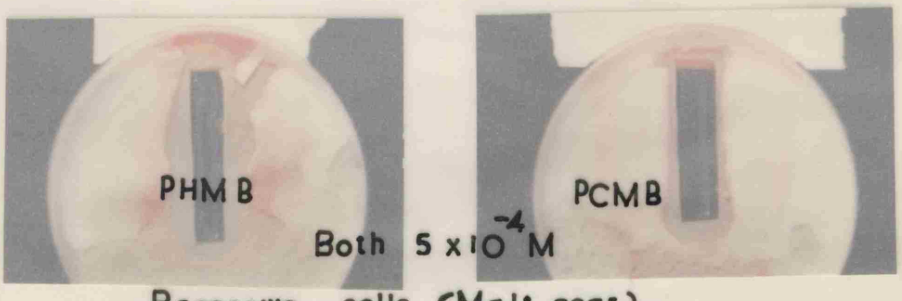


Upper view (Malt agar)

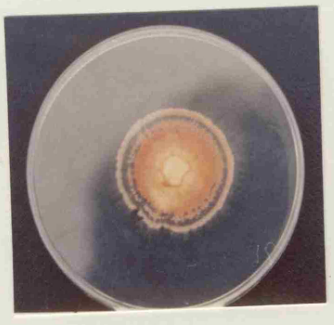
Lower view (Malt agar)



Lower view (Schopfer's agar)



Pregrown cells (Malt agar)



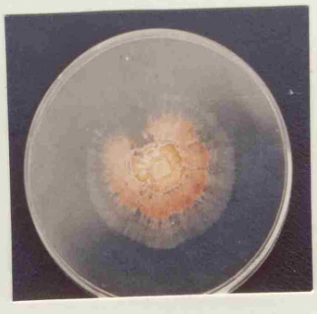
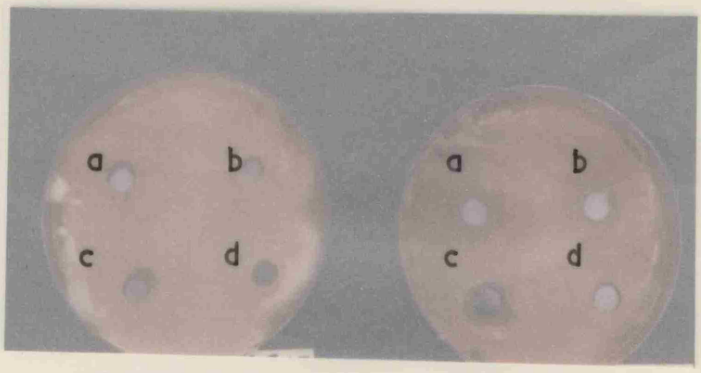
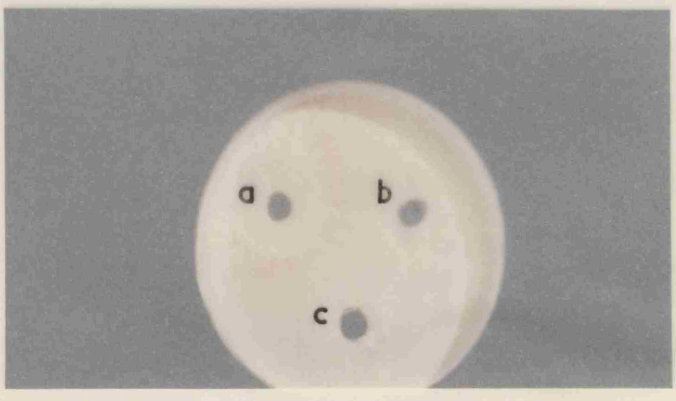


Plate IV



PHMB PCMB

Pregrown cells on Malt agar



PHMB

Pregrown cells on Schopfers agar

a 5×10^{-4} M

b 10^{-4} M

c 5×10^{-5} M

d 10^{-5} M

photomimetic compounds was unable to be transmitted to other cells not in contact with them. It might also appear that only the submerged mycelium and not the aerial hyphae and conidia were sensitive to these compounds. However, the most probable explanation is that whatever the immediate effects of these compounds are, then high molecular weight cell constituents are the likely sites of action.

Neither P-chloromercuriphenyl sulphonic acid nor p-hydroxymercuriphenyl sulphonic acid acted in the same way as PCMB and PHMB when incorporated into solid medium. Both these compounds are very specific -SH group inhibitors, and their failure to induce pigmentation of C.diospyri in the dark might suggest that -SH groups were not directly involved in the initial mechanism. As a further test, a range of the more commonly used -SH group inhibitors were also tested for their ability to act as photomimetic compounds with C.diospyri. (Table IX).

From these results, where none of the other compounds was effective, the situation resembles that found by Rau (1967) with F.aquaeductuum except that iodosobenzoic acid was slightly effective as a photomimetic compound. (13% that found with PHMB in liquid culture).

Cup plate methods were also employed and again only PHMB and PCMB were effective on both Malt agar and Schopfer's agar. PHMB appeared to inhibit the growth of C.diospyri to a greater degree than did PCMB, as measured by increase in colony diameter (Plate IV).

TABLE IX

-SH group inhibitors tested as photomimetic compounds in C.diospyri, grown on Malt agar plates for 7 days in the dark at 25°.

Compound test	Concentration range	Carotenoid production
Iodosobenzoic acid	5×10^{-3}M-5×10^{-6}M	none
n-ethylmaleimide	5×10^{-3}M - 5×10^{-6}M	none
iodoacetamide	5×10^{-3}M - 5×10^{-6}M	none
Mercury vapour	-	none

Although illustrating the response very well, the practical scope of working on solid media is very limited, and for further study of these compounds, in the light of the work of Rau (1967), it was hoped to use liquid medium. However, despite numerous attempts, no success was obtained with either PCMB or PHMB as photomimetic compounds in submerged culture. A series of experiments was performed making every effort to check out possible reasons for the non-activity of these -SH group inhibitors under these incubation conditions.

When spores were inoculated into Schopfer's or Malt broth in the presence of PCMB and PHMB (both in the range of concentrations of 5×10^{-4}M to 5×10^{-6}M) no carotenoid was detected after 10 days and the highest concentration used inhibited growth. To overcome this, spores inoculated into both media were allowed to grow for 5 days and then PCMB and PHMB (5×10^{-4}M to 5×10^{-6}M) added and the incubation continued for another 5 days. Although there was good growth, no carotenoid had been formed in conditions which held on agar plates.

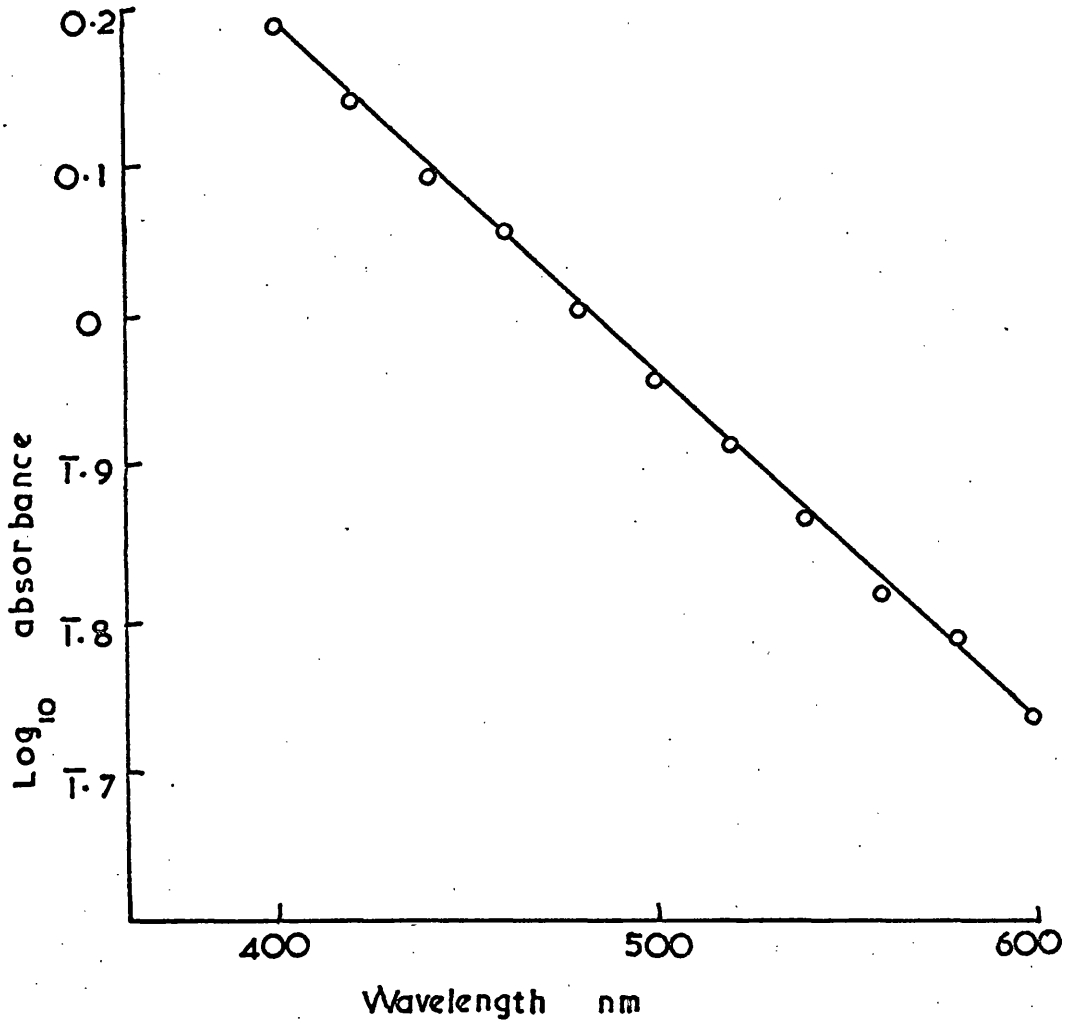
The possibility that over vigorous aeration and the insensitivity of 'conidia', the main growth form in submerged culture, were responsible, was eliminated by growing surface pellicles of C.diospyri in the dark on water flooded malt agar plates for 6 days, washing them aseptically in sterile distilled water, transferring them to fresh Schopfer's and malt broth containing the appropriate concentrations of PCMB and PHMB and allowing them to incubate under stationary and shake conditions for 10 days. In neither case was any pigment produced, nor was it when spores were allowed to grow in still culture in Schopfer's medium in the presence of inhibitors. The phosphate medium used by Rau (1967) was also unable to support light independent pigmentation under either aerated or still incubation conditions. The only partial success encountered was when cells were grown as surface pellicles on Malt broth (25ml/250ml conical flask). PCMB and PHMB both at concentrations

of 5×10^{-4}M - 5×10^{-5}M induced slight pigmentation of the fungus in the dark. However, the criticism levelled at solid media as a convenient experimental approach also applied to these conditions of growth. Because of this unexplainable difficulty, further experiments in this field were abandoned. None of the other -SH group inhibitors mentioned earlier was capable of substituting for light in submerged culture in both Schopfer's and phosphate medium in the same range of concentrations used on solid media.

During the course of these experiments, it was noticed that cells grown in the presence of PHMB (5×10^{-4}M) for 10 days in the dark in Schopfer's medium turned from a creamy white colour to a dark brown, suggesting the production of a melanin-like pigment. Identification of this pigment was obtained in the following manner. Mycelium was boiled under reflux in 0.5N sodium hydroxide for 30 min. Although the fungus was still coloured, repeating the extraction procedure did not remove much further pigment. A spectrum of the alkaline solution determined between 400nm and 600nm gave a straight line with a negative slope obtained by plotting the logarithm of the optical density against the wavelength (Fig 9). Determination of this slope gave a value of -0.0025 which compares favourably with that for a similar plot quoted by Schaeffer (1953) of -0.0027, and seemed to establish quite well the melanic nature of the pigment. A similar absorption spectrum of the culture filtrate which also appeared to contain the pigment gave a slope of -0.0021, again implying the production of melanin. Melanin has been used to describe a whole series of diversely coloured compounds mostly of unknown chemical structure formed by the action of a polyphenol oxidase. Why PHMB should induce this synthesis is not known, especially since no polyphenol oxidase activity could be detected in either light or dark grown cells under normal conditions of growth. Therefore, this would seem to rule out the possibility of subcellular disruption removing the geographical separation between the enzyme and its substrate caused by PHMB.

FIG.9

Absorption spectrum of an NaOH (0.5N) extract of cells of C.diospyri grown in the dark for 10 days at 25° in the presence of 5 x 10^{-4}M PCMB in Schopfer's medium.


0·2
0·1
0
$\bar{1}$·9
$\bar{1}$·8
$\bar{1}$·7
Log10 absorbance
400
500
600
Wavelength nm

Antimycin A.

Antimycin A shown by Batra (1967) to substitute for light in Mycobacterium marinum was tested for its ability to mimic light in carotenogenesis in C.diospyri. The results shown in Table X would suggest that Antimycin A was ineffective when incubated with dark grown cells of C.diospyri both in Schopfer's medium and phosphate medium as well as on a range of solid medium. Thus it appears that PCMB and PHMB and Antimycin A are not interchangeable as photomimetic compounds. Furthermore, rather than stimulate carotenoid production in the dark, Antimycin A at a concentration of 10^{-5}M inhibited pigment production in light grown cultures to the extent of approx. 90% of the control. Total inhibition of growth of C.diospyri in both the light and the dark was achieved with Antimycin A at a concentration of 5×10^{-5}M. Similar results were obtained with cells of C.diospyri incubated in phosphate medium.

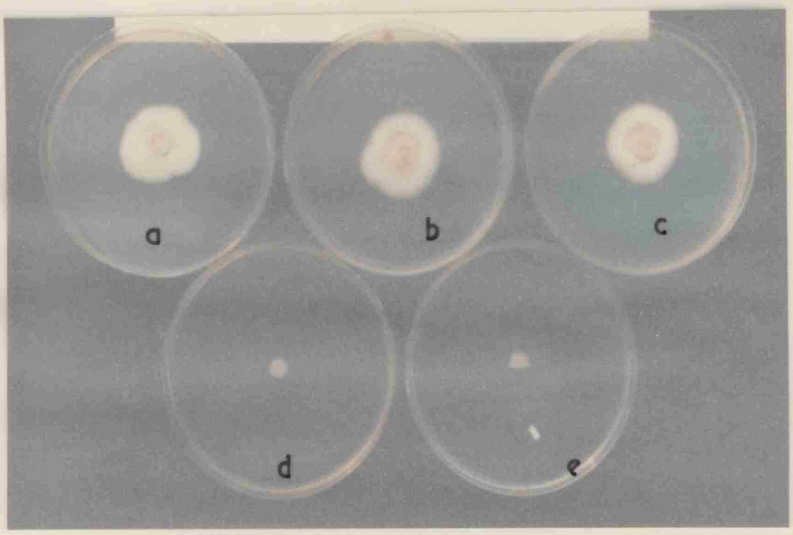
TABLE X

Effect of Antimycin A on Carotenoid production by C.diospyri grown in Schopfer's medium for seven days in the light and dark at 25^{o}.

Concentration of Antimycin A (Molarity)	Carotenoid Content % Control	
	Light	Dark
5×10^{-7}	98.8	nil
10^{-6}	99.2	nil
5×10^{-6}	71.0	nil
10^{-5}	<10 (poor yield)	nil
5×10^{-5}	no growth	no growth

Plate V

The effect of diphenylamine on carotenoid synthesis by C. diospyri grown at 25° in the light on Malt agar.



a 10^{-4} M

b 10^{-5} M

c 10^{-6} M

d 10^{-3} M

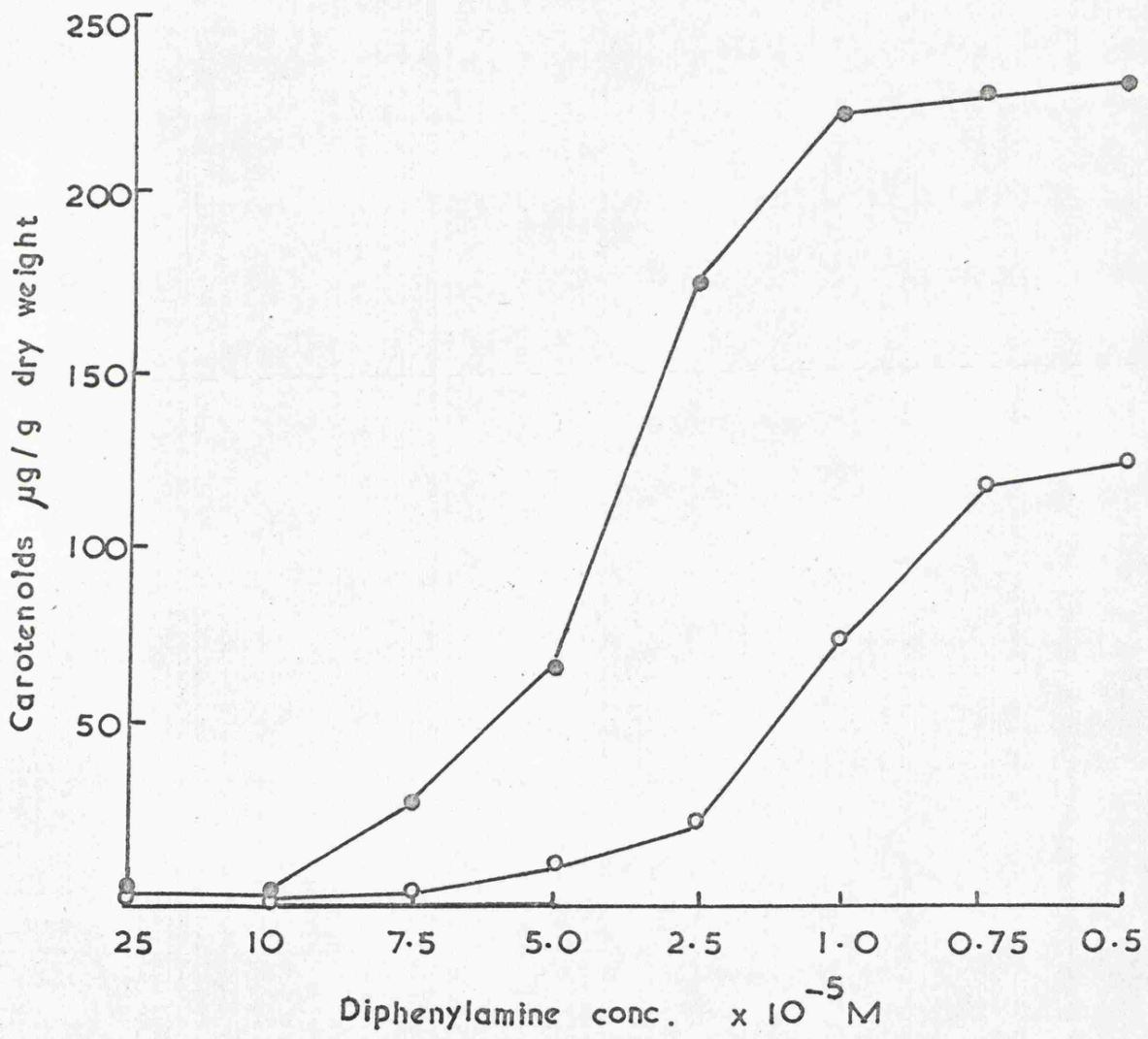
e 5×10^{-4} M

FIG.10

The effect of diphenylamine on carotenoid synthesis in C.diospyri grown under continuous illumination for 7 days. Pigment was estimated as described previously.

●—● Cells grown in Schopfer's medium.

○—○ Cells incubated in Phosphate medium.


250
200
150
100
50
Carotenoids µg/g dry weight
25
10
7·5
5·0
2·5
1·0
0·75
0·5
Diphenylamine conc. x 10^{-5} M

Hydrogen peroxide.

Because of the findings of Charlton (1953) that hydrogen peroxide would substitute for light in sporulation in Alternaria solani, and also for the reasons which will be discussed later, this compound was also tested with C.diospyri for its ability to substitute for light in carotenogenesis. Both fungal tissue grown on Malt agar and Schopfer's agar in petri dishes and in Schopfer's medium in submerged culture were incubated with hydrogen peroxide buffered to pH 5.8 in the range of concentrations 10^{-1}M to 10^{-4}M. Under none of these conditions of growth which gave good pigmentation in control cultures in the light did hydrogen peroxide induce carotenogenesis in the dark in C.diospyri after seven days.

(5). Inhibition of Carotenogenesis in C. diospyri.

(a) Diphenylamine.

Diphenylamine known to inhibit carotenogenesis in a wide range of biological material was also effective against both stationary and submerged cultures of C.diospyri (Plate V). Cells grown in the light in the presence of diphenylamine in both schopfer's medium and phosphate medium were analysed for carotenoid content and the results shown in Fig.10. Cells incubated in the phosphate medium appeared to be more sensitive to diphenylamine than those grown in Schopfer's medium i.e. approx 90% inhibition of pigment production in phosphate medium with 2.5 x 10^{-5}M diphenylamine as compared to approx. 12% inhibition in Schopfer's medium. This was taken to suggest that the actively growing fungus in the rich medium was able, partially at least, to reverse the effect of diphenylamine, whereas those cells grown in the phosphate medium were not. Also, cells grown in the presence of the inhibitor (10^{-4}M which allowed growth albeit at a lower amount, but totally inhibited carotenoid synthesis) for 6 days in the light, when harvested, washed free of diphenylamine with sterile 10% (v/v) ethanol and resuspended in

diphenylamine free medium produced carotenoids to the full extent after further growth of 6 days in the light.

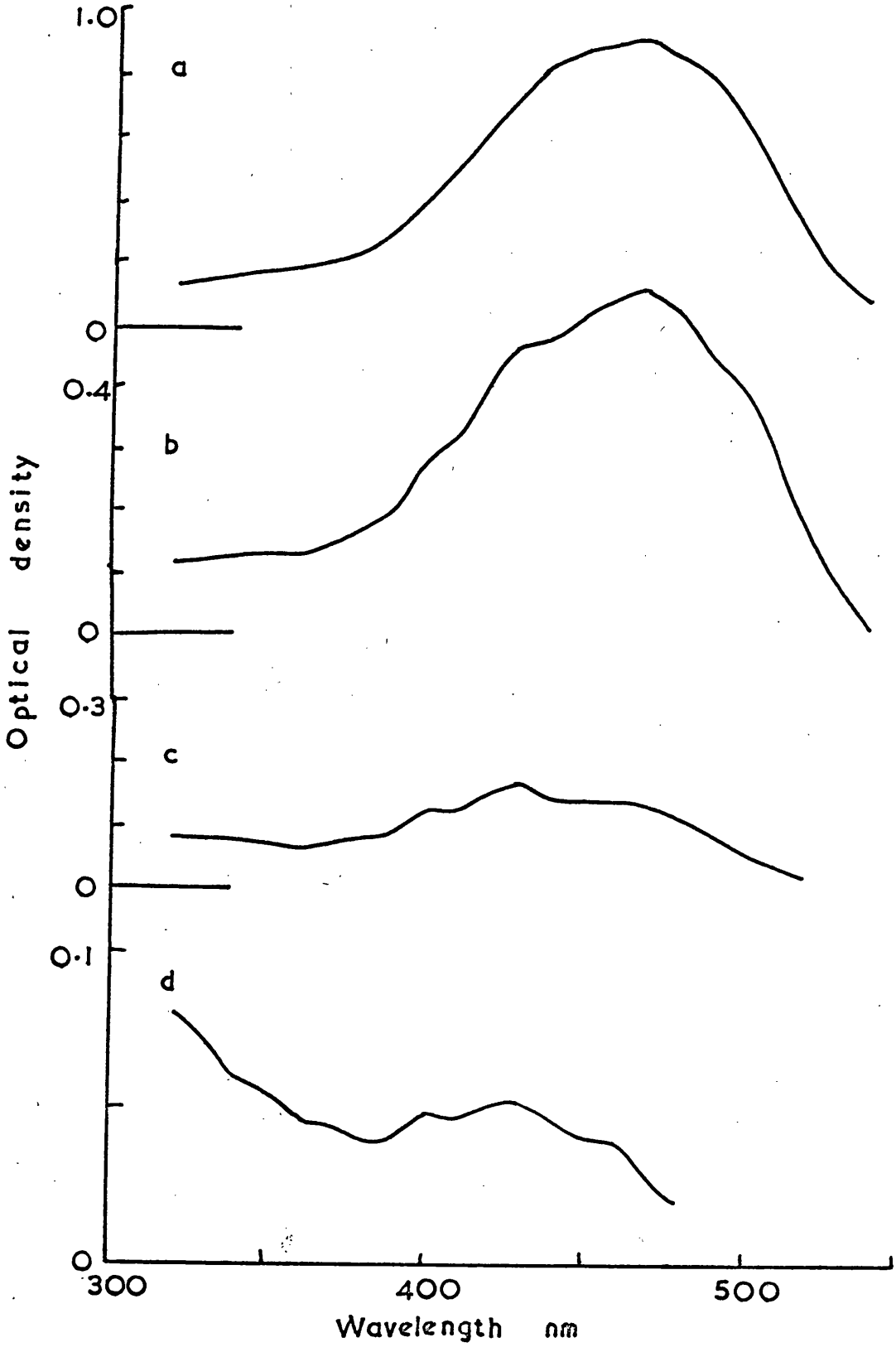
Although carotenoid content of diphenylamine treated cells was not analysed, as well as quantitative changes, diphenylamine also appeared to alter qualitatively the carotenoid composition of C.diospyri. Absorption spectra were recorded of extracts of cells grown in the range of concentration of inhibitor as shown in Fig II.. By comparison with the spectrum of normal cells (see Fig IV), there would appear to be a definite shift of peaks towards the shorter wavelengths, suggesting that this inhibitor was preventing the synthesis of the more unsaturated carotenoids. The major peak at 475nm found in normal light grown cells had nearly disappeared at 5×10^{-5}M diphenylamine to be replaced by smaller maxima at 430nm, 400nm and 370nm. Extracts from cells grown in 7.5×10^{-5}M diphenylamine also possessed these peaks with the appearance of another at 350nm due possibly to the presence of phytofluene.

Diphenylamine appeared only to inhibit the synthesis of carotenoids after the initial light dependent stage. Cells incubated in phosphate medium were photoinduced in the normal way. 7.5×10^{-5}M diphenylamine was added 30 min before, and after 90 min. illumination and the carotenoid content of the cells calculated after 3 days incubation in the dark. Where required, diphenylamine was removed from the cells by harvesting them under dim red light, washing with 10% (v/v) ethanol and then resuspending them in fresh phosphate medium for the dark incubation. Results shown in Table XI indicate that only when diphenylamine was present during the dark incubation was pigment production inhibited.

FIG.11

Absorption spectra of carotenoids from cells of C.diospyri grown in the presence of diphenylamine under continuous illumination at 25^{o} in Schopfer's medium. Pigments estimated as previously described.

(a) $10^{-}5$ diphenylamine
(b) 2.5×10^{-5}M diphenylamine
(c) 5×10^{-5}M diphenylamine
(d) 7.5×10^{-5}M diphenylamine


1.0
a
0
0.4
b
0
0.3
c
0
0.1
d
0
Optical density
300
400
500
Wavelength nm

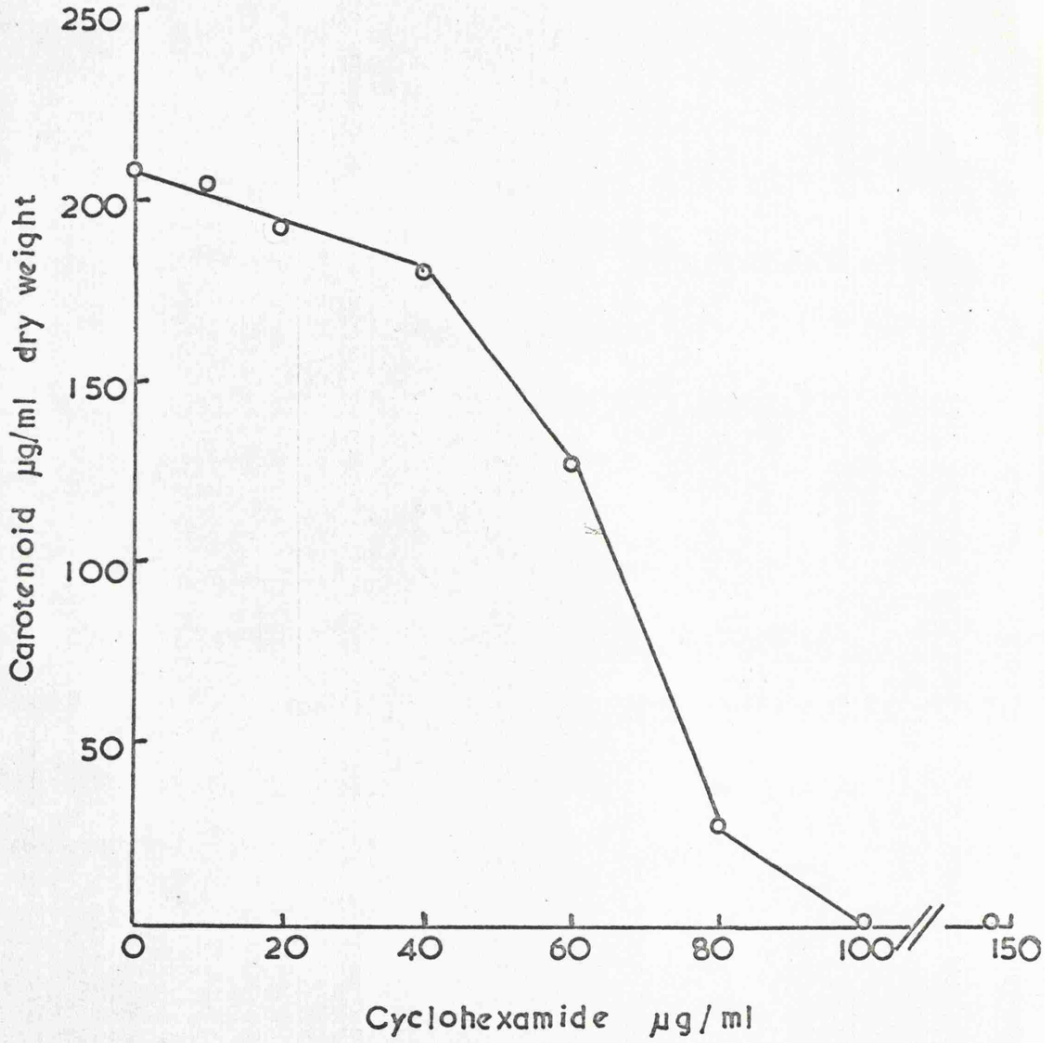

250
200
150
100
50
Carotenoid µg/ml dry weight
0
20
40
60
80
100
150
Cyclohexamide µg/ml

FIG.12

The effect of cyclohexamide on carotenoid production by cells of C.diospyri grown under continuous illumination at 25° for 7 days in Schopfer's medium. Pigments estimated as previously described.

TABLE XI

Effect of Diphenylamine on the stages of carotenoid production by C.diospyri. Method as described in the text.

Light Induction	Dark Incubation	Carotenoids
Presence of Diphenylamine (7.5×10^{-5}M)		µg/g.dry wt cells
+	+	nil
+	-	31.7
-	+	nil
-	-	49.5

(b). Cyclohexamide and Chloramphenicol.

Because the overall production of carotenoids in C.diospyri seemed to resemble the processes described for N.crassa and F.aquaeductuum, where de novo protein synthesis has been suggested to occur (Harding & Mitchell, 1969; Rau, 1967) it was decided to investigate the effects of known inhibitors of protein synthesis on pigment production in C.diospyri. The possible temperature dependence of this process after the initial photochemical oxidation was also taken as a suggestion that protein synthesis was necessary.

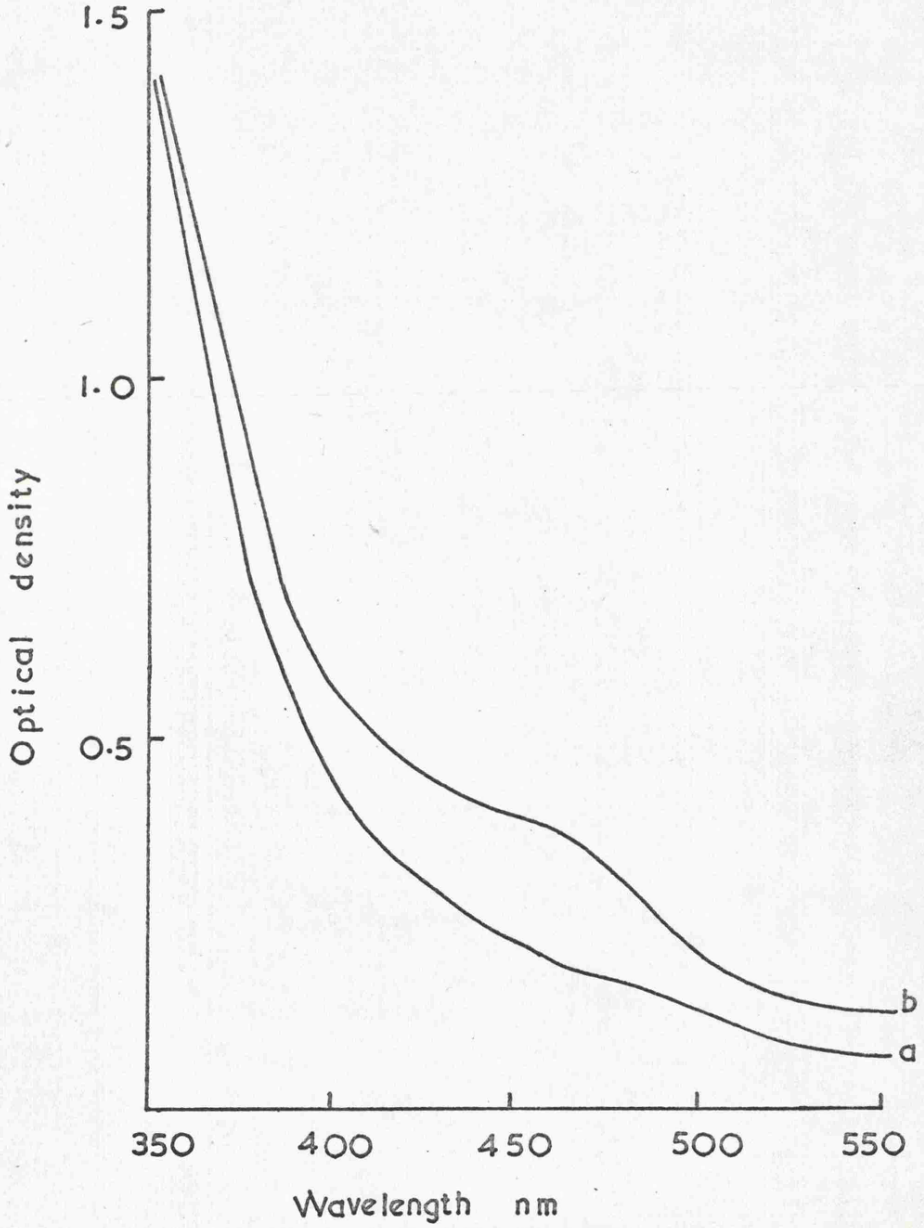
Cyclohexamide is thought to inhibit protein synthesis by preventing the formation of polypeptides on the ribosomes in eukaryotic cells. Its effect upon carotenogenesis in cultures of C.diospyri grown in Schopfer's medium is shown in Fig 12. From this data, cyclohexamide inhibited pigment production completely at a concentration of 100µg/ml medium, but in most of the experiments performed with this compound there was also severe inhibition of growth as total dry weight at this level. There was only very slight reduction in carotenoid content of cells up to 40µg/ml medium. The range of concentration of inhibitor which were effective were much higher than those found to inhibit totally carotenoid synthesis in both N.crassa and F.aquaeductuum (2µg/ml). It is quite possible that cells of C.diospyri are relatively impermeable to the cyclohexamide. No results were obtained

FIG.13

Absorption spectra of filtrates of cells grown in the light and dark in Schopfer's medium. The solutions were treated successively with 3% (w/v) potassium permanganate solution followed by 3% H_2O_2.

(a) Light

(b) Dark


1·5
1·0
0·5
Optical density
b
a
350
400
450
500
550
Wavelength nm

to determine which stage in the photoinduction mechanism was affected by cyclohexamide, because experiments performed with the inhibitor and phosphate medium were not successful due to difficulties encountered with obtaining cell quantities suitable for analysis. There is an obvious need for further study with this compound and carotenoid production by C.diospyri, but this indicates that de novo protein synthesis might be one step in the overall process.

Chloramphenicol did not inhibit carotenogenesis in C.diospyri. in both Schopfer's and phosphate medium in a final concentration up to 40µg/ml. Growth also appeared to be unaffected as measured by total dry weight production

Mepacrine, suggested to be an inhibitor of flavoprotein synthesis did not inhibit carotenogenesis in light grown cultures of C.diospyri in Schopfer's medium in the range of concentration of O-1mM. Lyxoflavin, another flavin inhibitor was not tested because of failure to obtain any of this chemical commercially.

(6). Production of Extracellular Riboflavin by C.diospyri.

During the course of this investigation, it was noticed that the culture filtrates of dark grown cells were strongly fluorescent, whereas those from light grown cells were much less so. Because of the involvement of riboflavin as a photoreceptor in fungi, and the possibility that this fluorescence might be due to riboflavin, the identity of this material produced by C.diospyri in Schopfer's medium was sought. Preliminary attempts were confined to visible spectra of culture filtrates and typical ones obtained are shown in Fig.13. The slight peak present in the sample from dark grown cells at 450nm could have been due to riboflavin, and it was absent in spectra of culture filtrates from light grown cells, which incidentally closely resembled the visible spectra of uninoculated medium. However, because of interfering material no clear definition was possible in the ultra violet region.

FIG.14.

Emission spectra of culture filtrates (Schopfer's medium) from light and dark grown cells of C.diospyri. Samples were prepared as described in Materials and Methods.

Wavelength of exciting light was 380nm.

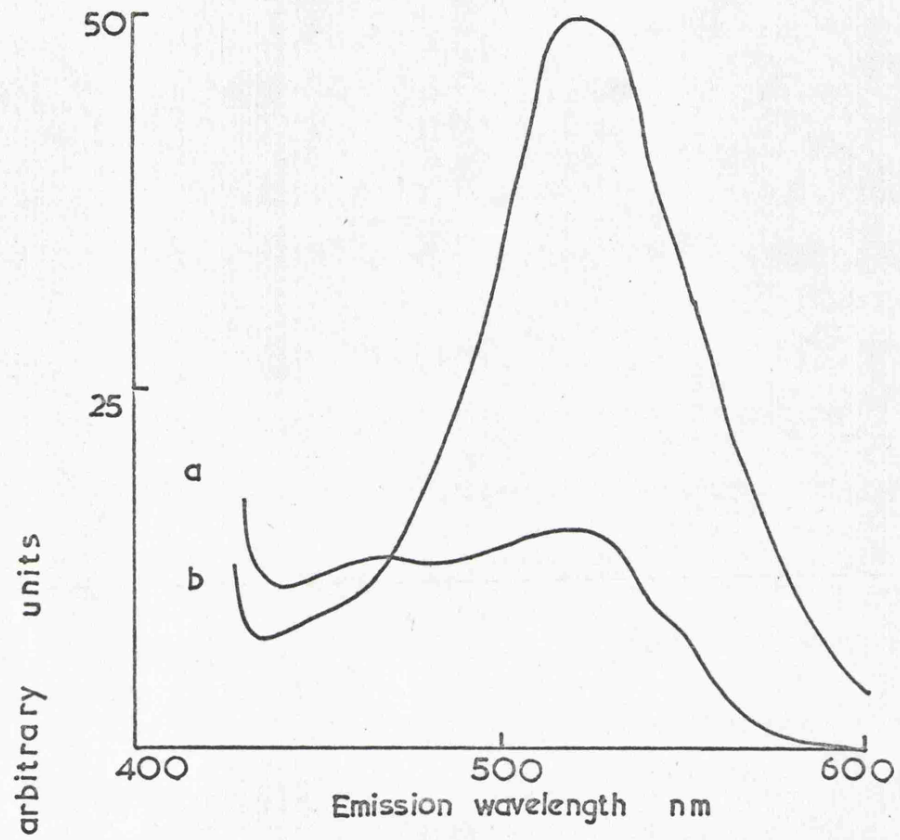
(a) Light

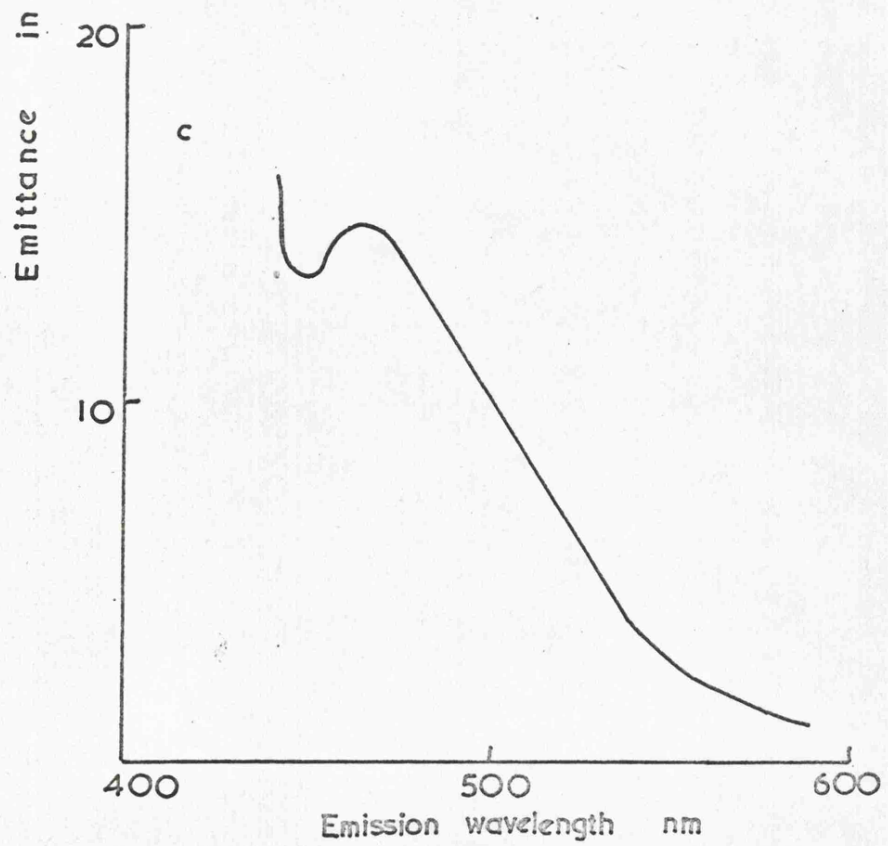
(b) Dark

FIG.14a.

Emission spectrum of uninoculated Schopfer's medium. Sample was prepared as previously described.

Wavelength of exciting light was 380nm.


50
25
a
b
400
500
600
Emission wavelength nm
Emittance in arbitrary units


20
10
c
400
500
600
Emission wavelength nm

A study of fluorescent spectra of culture filtrates was considered a better experimental approach, and Fig. 14 shows the typical curves obtained. Comparison of the spectrum obtained from culture filtrates from dark grown cells with that of authentic riboflavin (Fig.17), illustrates their large similarity. Filtrates from light grown cultures lacked this sharp peak at 530nm with the appearance of a smaller plateau at 470-480nm, also found in uninoculated Schopfer's medium (Fig 14a).

Before proceeding further, conditions for the measurement of riboflavin on the spectrophoto fluorometer were studied. Regardless of the wavelength of exciting light, riboflavin always showed an emittance peak at 530nm. The variation of exciting light with emittance is shown in Fig 15. Thus, for all quantitative measurements, a wavelength of 380nm was used for the exciting light. Riboflavin estimations were performed from the standard curve shown in Fig. 16.

Chromatographic analyses of filtrates from dark grown cultures revealed the presence of one major compound with an Rf of 0.36 corresponding to that of riboflavin (Plate VI). Elution of this component with 3ml hot water, gave a yellow solution with the absorption spectrum shown in Fig.18. Maxima at 450, 375, 280nm, the same as riboflavin in acid solution, confirmed that the fluorescent material produced by C.diospyri was riboflavin.

Culture filtrates from light grown cells, on the other hand, when subjected to chromatographic analysis in the same way as those from dark grown cells showed the presence of one major component which gave a blue fluorescence, and had an Rf of 0.68. This suggested lumichrome the acidic photodegradative product of riboflavin. It was partially identified by chromatography of standard lumichrome and lumiflavin, prepared by exposing solutions of riboflavin at pH 4.5 and 8.0 to a 40 watt fluorescent light for 6h and extracting the photolysis compounds with chloroform.

FIG.15.

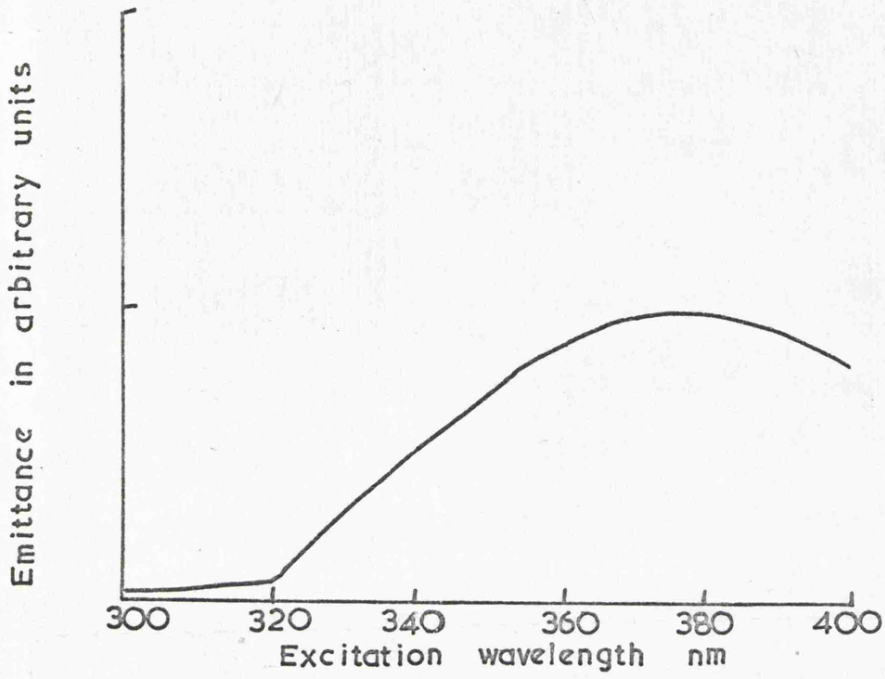
Variation in emittance with wavelength of excitation light of a solution (1μg/ml) of riboflavin. Emission wavelength was 530nm.

FIG.16

Standard curve for riboflavin estimations. Method of preparing samples was as previously described.

Excitation wavelength of 380nm.

Emission wavelength of 530nm.


Emittance in arbitrary units
300
320
340
360
380
400
Excitation wavelength nm

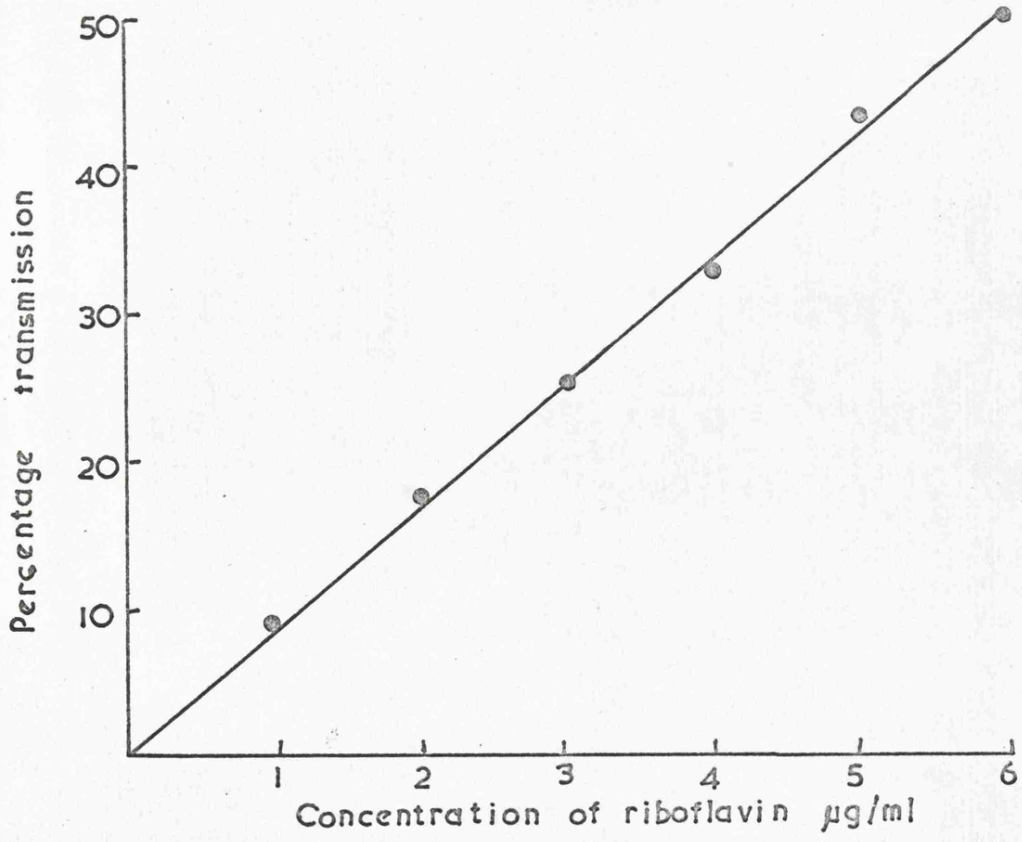

Percentage transmission
50
40
30
20
10
1
2
3
4
5
6
Concentration of riboflavin µg/ml

FIG. 17.

Emission spectrum of authentic riboflavin (1μg/ml) in aqueous solution. The sample was prepared as described previously.

Excitation wavelength was 380nm.

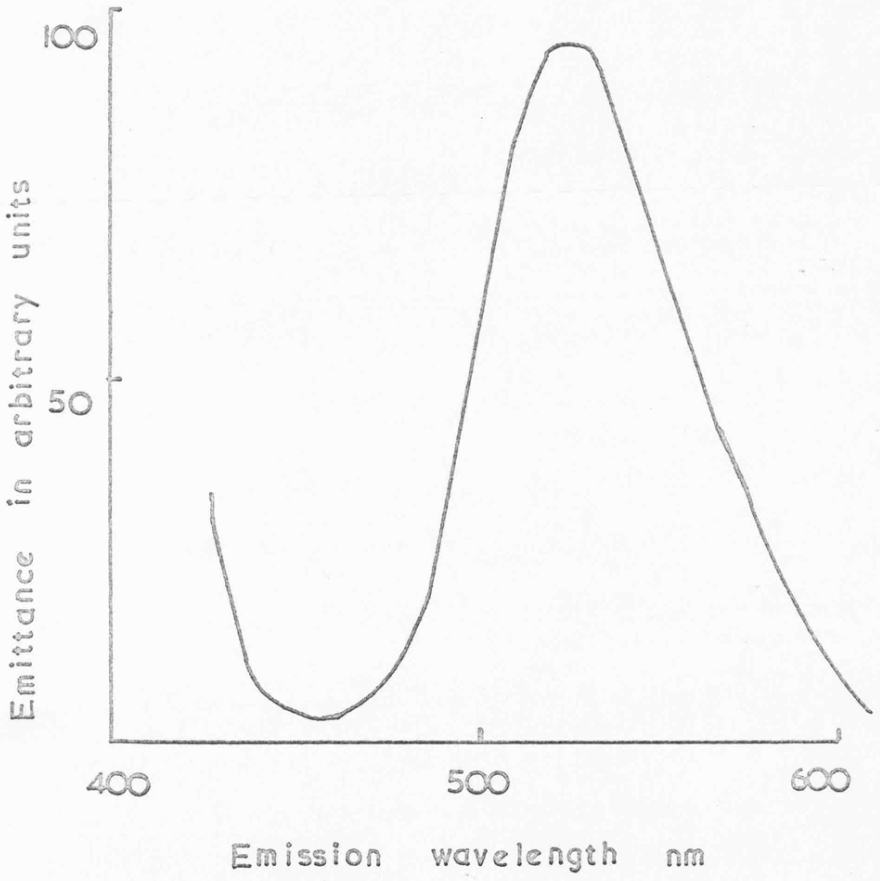
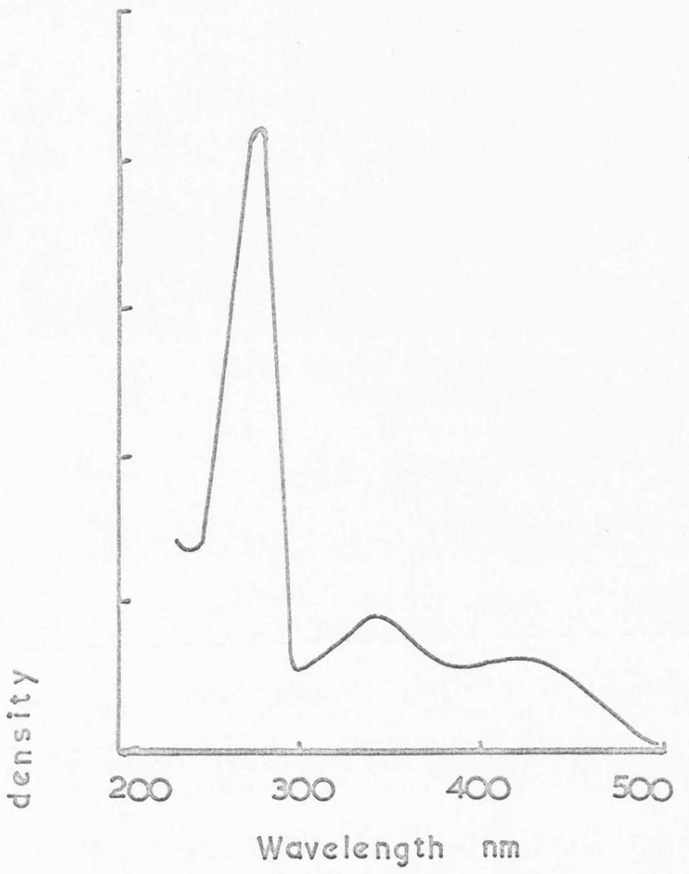

100
50
Emittance in arbitrary units
400
500
600
Emission wavelength nm

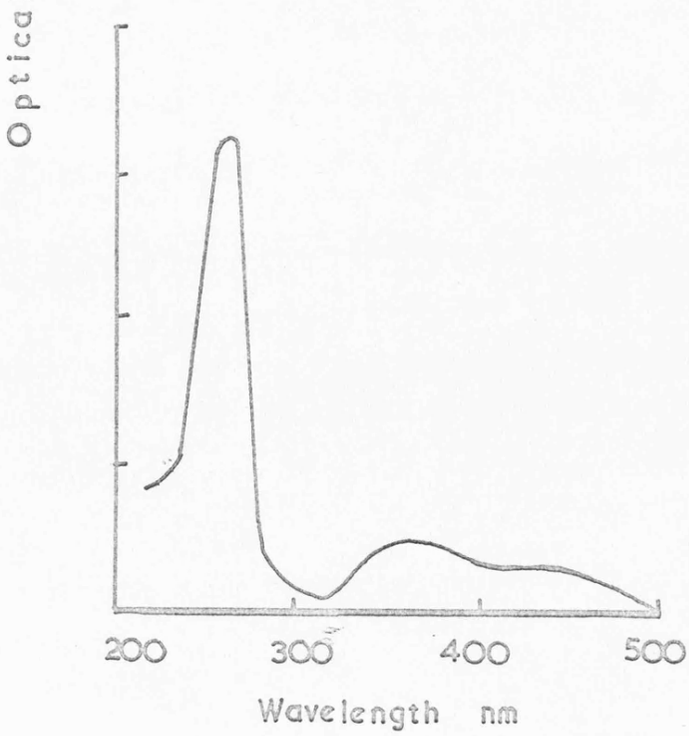
FIG. 18

Absorption spectrum of compound eluted from the chromatogram of culture filtrates from dark grown cells. The solution was read in aqueous solution pH 5.0. Method for separation as described in the text.

FIG. 19.

Absorption spectrum of compound eluted from the chromatogram of culture filtrates from light grown cells. Method as described in text. The spectrum was measured in 0.1N NaOH solution.


Optical density
200
300
400
500
Wavelength nm


Optical density
200
300
400
500
Wavelength nm

Lumichrome prepared in this manner fluoresced blue under ultra violet light and had an Rf of 0.69 when chromatographed under the same conditions as the samples. Lumiflavin, when chromatographed had an Rf of 0.47 and a yellow fluorescence. Standard lumichrome and the test compound ran together as a single component in the solvent system used for chromatography. On elution of the sample with 5ml 0.1N sodium hydroxide, the resulting pale yellow solution had an absorption spectrum as shown in Fig.19, when read against a blank prepared by extracting in the same manner a piece of chromatography paper. This spectrum is in good agreement with that obtained by McNutt (1954) for lumichrome. There also appeared to be traces of riboflavin present on chromatograms of culture filtrates of light grown cells of C.diospyri, but no other flavin compound could be found in either sample.

Riboflavin production by C.diospyri.

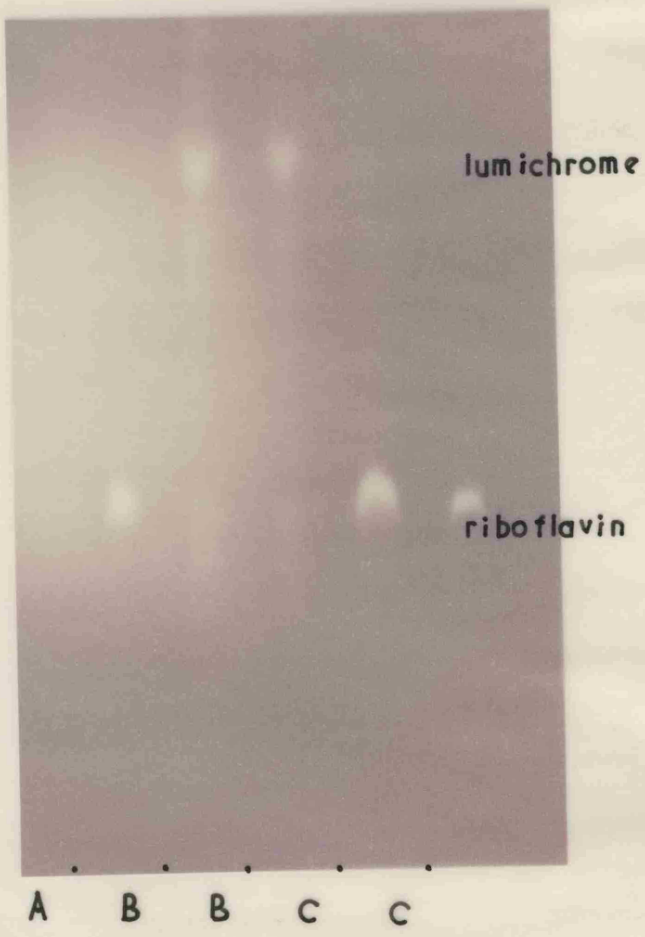
Culture filtrates were analysed for riboflavin over a period of 12 days growth in Schopfer's medium and the results shown in Fig.20. The graph illustrates the production of up to 3.5 μg/ml free riboflavin in filtrates from dark grown cultures. Light grown culture filtrates contained 0.7 μg/ml riboflavin after four days with a subsequent decrease, presumably due to photodestruction of riboflavin. This photodestruction in the light exposed culture filtrates was further investigated and the results shown in Fig.21. Cells grown in the light when replaced in darkness immediately started to accumulate riboflavin in the culture filtrates, suggesting that the biosynthetic capacity for riboflavin was unimpared in light grown cultures, and the failure to detect riboflavin in filtrates from them was due to the equimolar formation of lumichrome from riboflavin on exposure to light. It can be seen (Fig.21) that the destruction of riboflavin in the light was fairly rapid, over 70% after 24h, and lumichrome was the only product detected chromatographically throughout the exposure experiment. Carotenoid production in dark grown cells subsequently exposed to light visibly commenced after about

Plate VI

Chromatography of culture filtrates of light and dark grown cells of C. diospyri. Method as described in the text.

Paper photographed under uv light C 254nm)

Film exposed for 5 min.



A - Riboflavin standard

B - Filtrates from light grown cultures

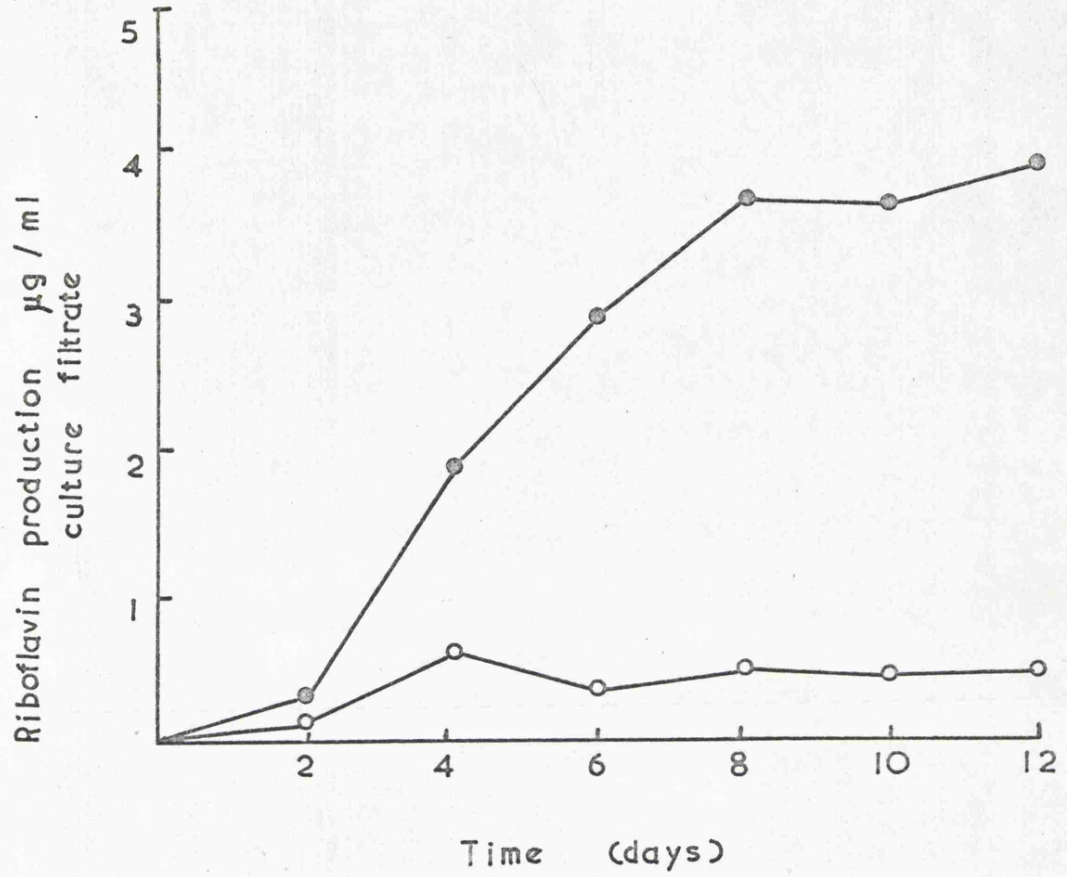
C - Filtrates from dark grown cultures

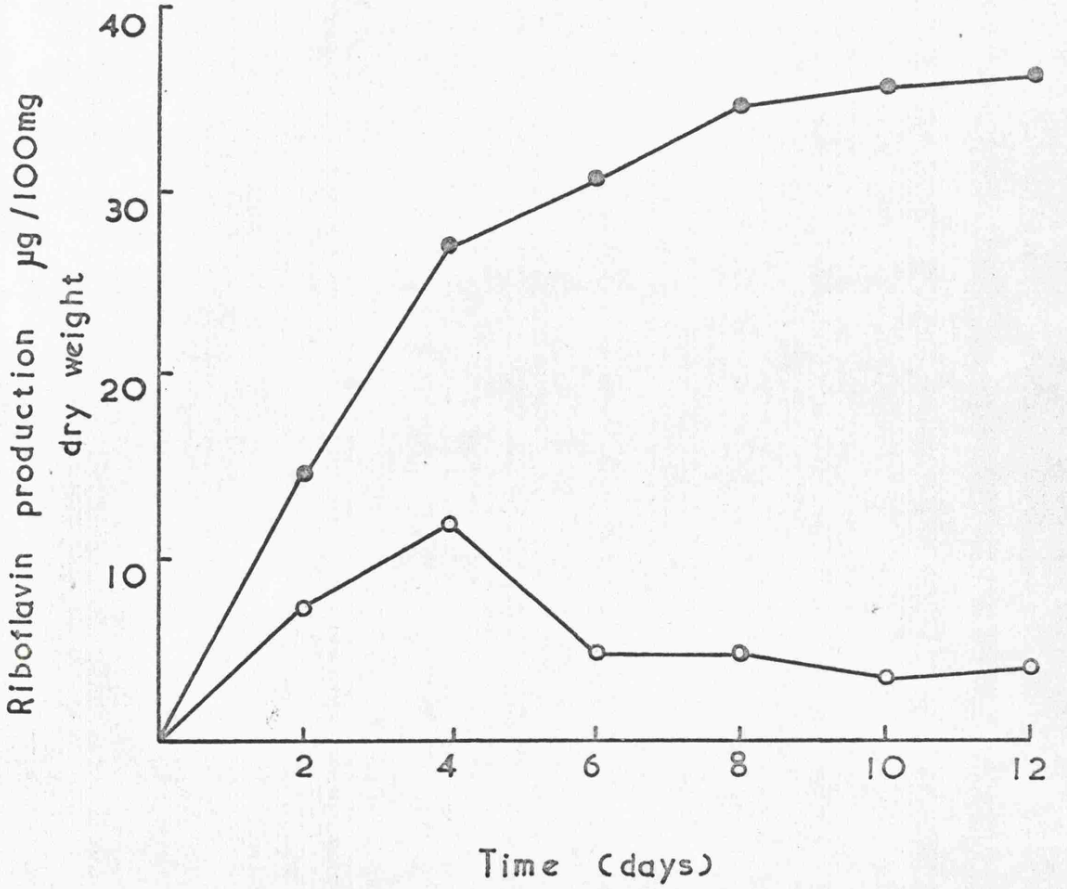
FIG. 20.

The production of extracellular riboflavin by C.diospyri in Schopfer's medium in submerged culture at 25°.
Results are expressed as both μg/ml riboflavin and μg/g. dry weight of cells.

●—● Dark

○—○ Light


Riboflavin production µg / ml culture filtrate
5
4
3
2
1
2
4
6
8
10
12
Time (days)


Riboflavin production µg /100mg dry weight
40
30
20
10
2
4
6
8
10
12
Time (days)

24h when nearly all the riboflavin had been photolysed to lumichrome. This observation might imply that lumichrome was acting as an inducer for pigment synthesis possibly by competitive inhibition with riboflavin (Hopkins, 1939). However, dark grown cells suspended in fresh Schopfer's medium containing lumichrome in the same amount as in 6 day old light grown culture filtrates did not on subsequent incubation in the dark produce any carotenoid pigments. This may have been due to an excess production of riboflavin by these cells in the dark overcoming any antagonistic properties of lumichrome. Another point to consider is that if lumichrome were involved in the photoinduction of pigments, its effect might be indirect rather than direct. The addition of lumichrome to the growth medium of dark grown cells did not appear to interfere with the subsequent synthesis of riboflavin by them.

Riboflavin content of cells.

Cells grown in Schopfer's medium in the light and dark were harvested and their riboflavin content analysed. Results in Fig.22 indicate that this reached a maximum after 4 days growth and then decreased to a constant level after 10 days. There was no difference in riboflavin content between cells grown in the light and dark, results which agree with those obtained by Zalokar (1957) for light and dark grown cultures of N.crassa.

As one of the aims of this project was to investigate possible photoreceptors, aqueous extracts of light and dark grown cells of C.diospyri were examined for pigments other than carotenoids which might have a role in the reception of the light signal. An absorption spectrum of a 75% (w/v) methanolic extract from dark grown cells which was slightly yellow in colour is shown in Fig.23a. This spectrum was very much like that obtained by Trinci and Banbury (1969) from dark grown cultures of Aspergillus giganteus, and suggested by them to resemble chrysogenin a pigment produced by P.chrysogenum and anthraquinoid in nature.

FIG. 21.

Subsequent fate of riboflavin production in submerged culture of C.diospyri. Cultures were grown for 5 days in the light and the dark at 25° and then the conditions reversed.

O—O Cultures grown in the dark then exposed to light.

●—● Cultures grown in the light then replaced in darkness.

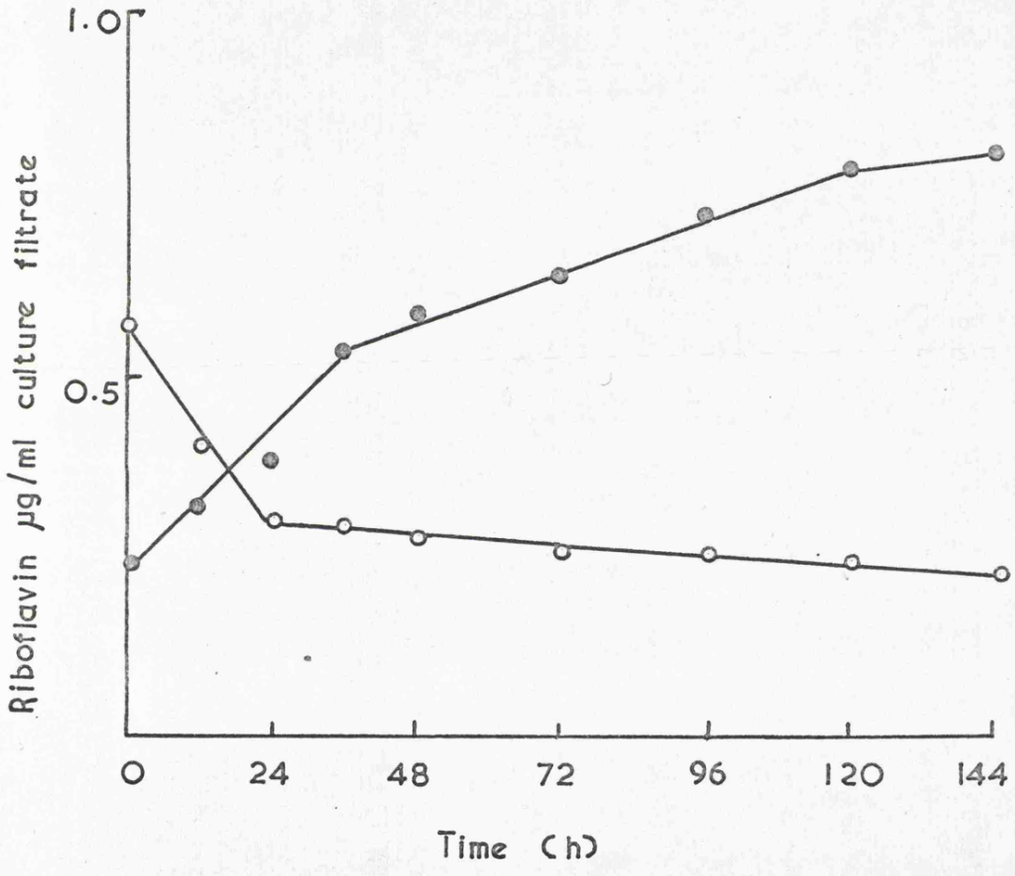
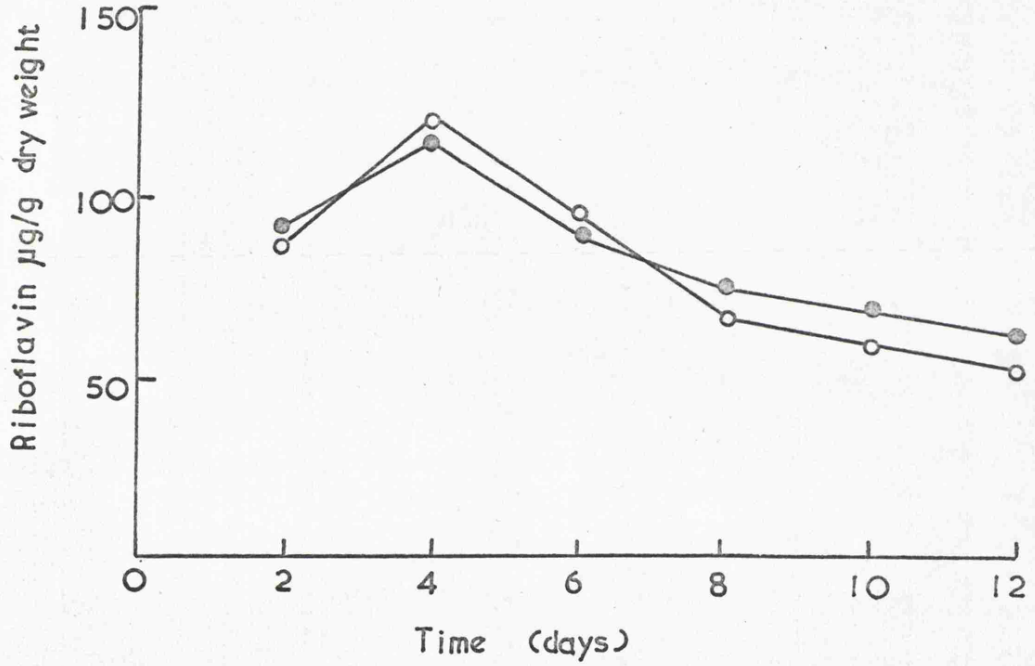

1.0
0.5
Riboflavin µg/ml culture filtrate
0
24
48
72
96
120
144
Time (h)

FIG. 22

Riboflavin content of cells of C.diospyri grown in submerged culture in the light and dark in Schopfer's medium at 25°.

O—O Light grown cells

●—● Dark grown cells.


Riboflavin µg/g dry weight
150
100
50
0
2
4
6
8
10
12
Time (days)

The fluorescence spectrum of this extract from C.diospyri (Fig.23b) however, possessed a sharp maximum very similar to that obtained for riboflavin, and unlike the fluorescence spectrum for chrysogenin (Wolf, Kim & Jones, 1960). Chromatography of this extract gave a major component with an Rf of 0.36 corresponding again to that for riboflavin. Therefore it is quite possible that the pigment described by Trinci & Banbury (1969), was flavin in nature, even riboflavin, whose characteristic absorption spectrum was blurred by other interfering material. Similar fluorescence spectra were obtained from hot water extracts of dark grown cells and light grown cells. This might suggest that the water soluble pigment could not be acting as the photoreceptor substance, and also might seem to indicate that C.diospyri contained an appreciable amount of free riboflavin intracellularly, not all being bound as flavin nucleotides or flavoproteins. In no instance throughout this investigation was any lumichrome detected in light grown cell extracts.

Inhibition of Riboflavin production.

Diphenylamine an inhibitor of carotenogenesis in C.diospyri was also found to inhibit the production of riboflavin in culture filtrates of dark grown cells in the same range of concentration as those which inhibited carotenogenesis in cells grown in the light in Schopfer's medium (Fig.24). These results show that 10^{-4}M diphenylamine almost completely inhibited riboflavin production, whereas 10^{-5}M diphenylamine allowed the extracellular riboflavin content to reach the same level as the control. To ensure that this result was due wholly to inhibition of synthesis and not a chemical destruction or alteration of riboflavin, an aqueous solution of riboflavin (4μg/ml) was incubated in the dark for 6 days in the presence of 10^{-4}M diphenylamine. Fluorometric analysis, both quantitative and qualitative (fluorescent spectra), revealed that there was no detectable loss of riboflavin over the control incubated without diphenylamine. Analysis of this mixture and the samples chromatographically failed to

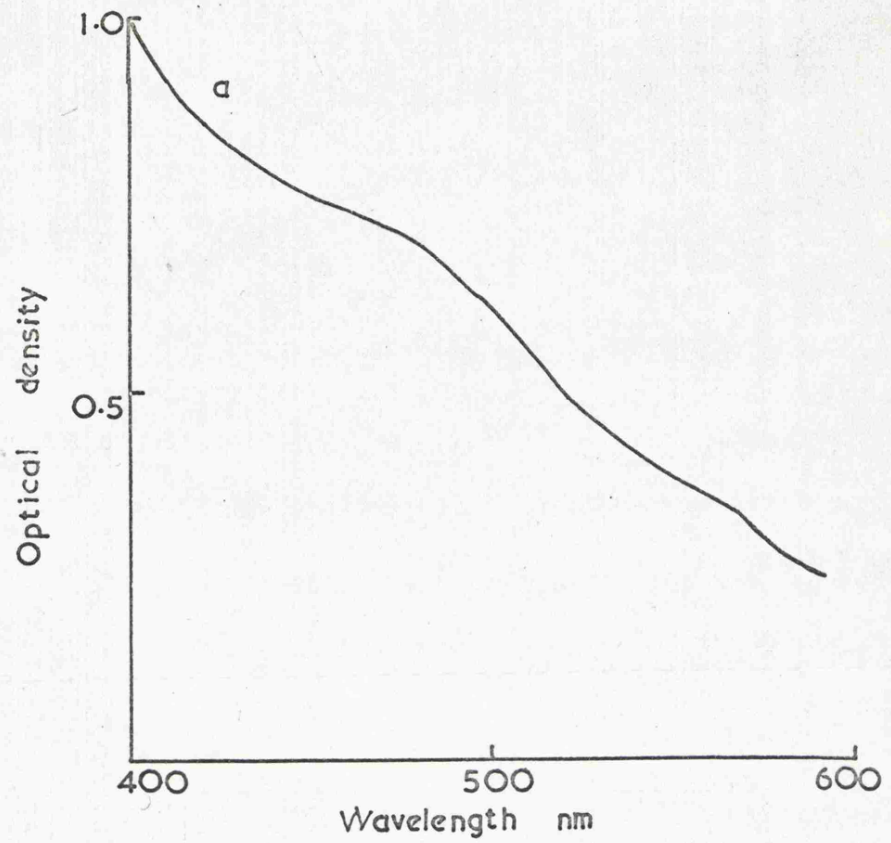
FIG. 23(a).

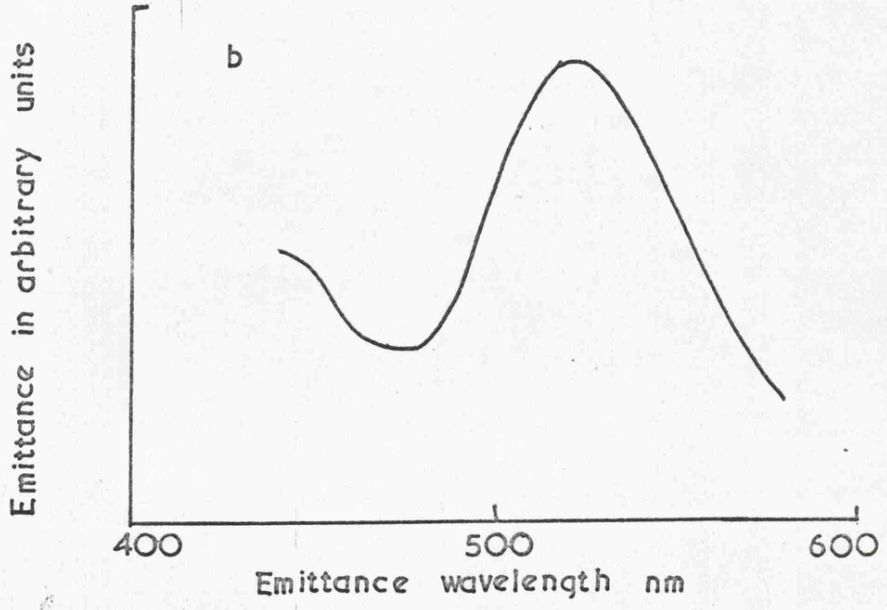
Absorption spectrum of a 75% (v/v) methanolic extract of dark grown cells of C.diospyri from Schopfer's medium.

FIG.23(b).

Emission spectrum of a 75% (v/v) methanolic extract of C.diospyri grown in the dark at 25^{o} in Schopfer's medium.

Excitation wavelength was 380nm.


1·0
0·5
a
Optical density
400
500
600
Wavelength nm


b
Emittance in arbitrary units
400
500
600
Emittance wavelength nm

detect any lumichrome or lumiflavin or any other fluorescing components. The fluorescence spectra of culture filtrates from cells grown in the presence of diphenylamine also supports the evidence of a direct effect of diphenylamine on riboflavin production (Fig.25).

Analysis of cells grown in the presence of diphenylamine gave the results shown in Table XII. This also shows that diphenylamine had altered the riboflavin content of the fungus, presumably by interfering with its synthesis and the effect appears to be more pronounced in dark grown cells. Whether the change in riboflavin synthesis induced by diphenylamine was the direct cause of inhibition of carotenoids has not been determined, but its possible significance will be discussed later.

TABLE XII.

Riboflavin content of cells of C.diospyri grown in the presence of diphenylamine for 6 days at 25° in Schopfer's medium.

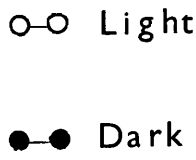
	Riboflavin content (μg/g.dry weight)	
Diphenylamine concentration (Molarity)	Light grown cells	Dark grown cells
10^{-4}	65	52
7.5×10^{-5}	70	58
5.0×10^{-5}	80	82
2.5×10^{-5}	79	77
10^{-5}	76	80
Blank	84	82

Cyclohexamide, also shown to inhibit carotenogenesis possibly in a different fashion, was found not to alter the production of riboflavin in filtrates from dark grown cells incubated with the inhibitor in the range of concentrations of 0-100μg/ml of Schopfer's medium.

The effect of mepacrine on riboflavin production by C.diospyri was also investigated. Because both these compounds are strongly fluorescent, the culture filtrates were examined chromatographically

FIG. 24

The effect of diphenylamine on the extracellular production of riboflavin by C.diospyri. Cells were grown for 7 days at 25° in Schopfer's medium and riboflavin estimated as described previously.



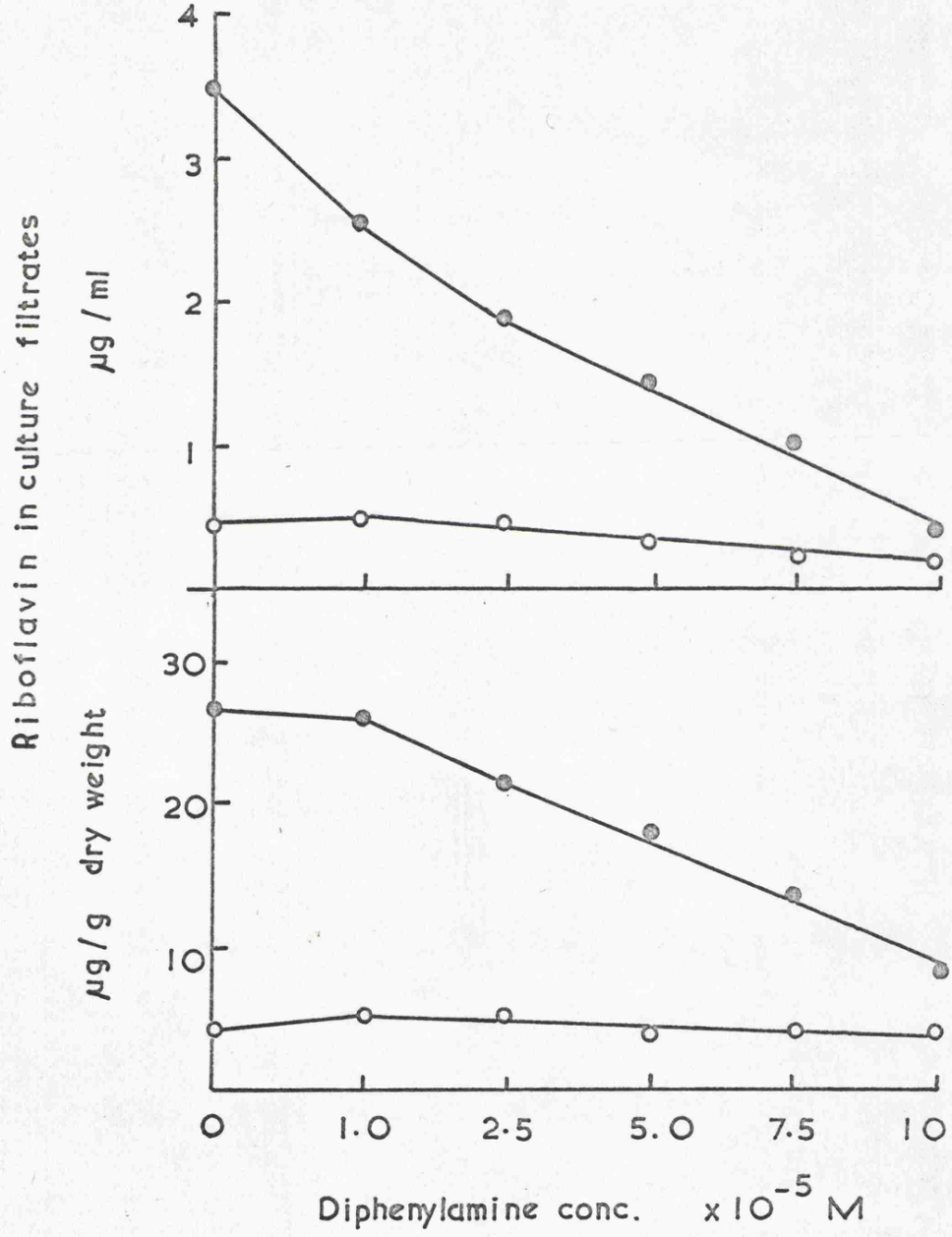

Riboflavin in culture filtrates
µg / ml
µg/g dry weight
4
3
2
1
30
20
10
0
1.0
2.5
5.0
7.5
10
Diphenylamine conc. x 10⁻⁵ M

FIG. 25.

Emission spectra of culture filtrates from cells grown in the presence of diphenylamine in Schopfer's medium. Samples were analysed as described previously.

Excitation wavelength was 380nm.

A: light grown culture filtrates

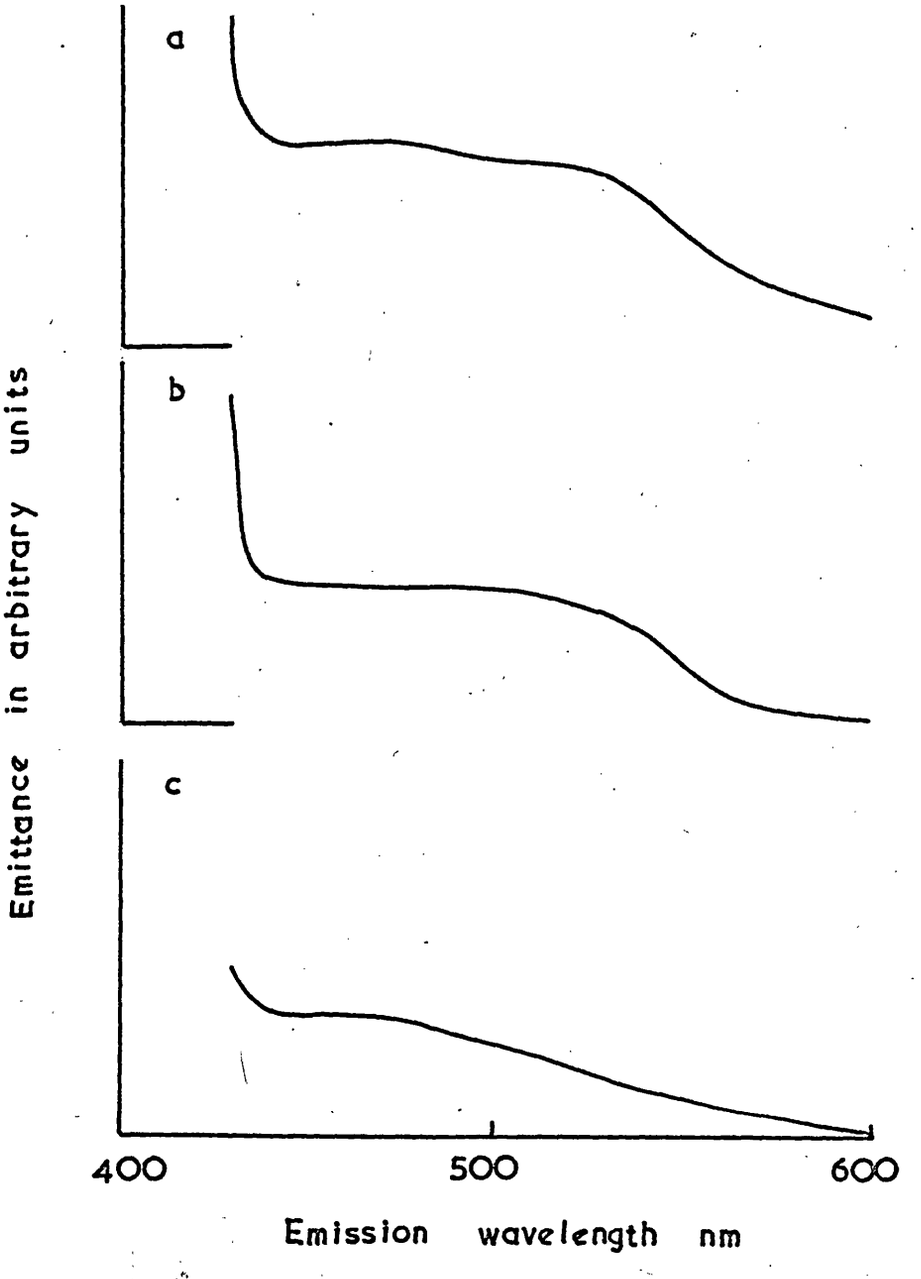
B: dark grown culture filtrates

(a) 10^{-5}M

(b) 5×10^{-5}M

(c) 10^{-4}M

A


a
b
c
Emittance in arbitrary units
400
500
600
Emission wavelength nm

B

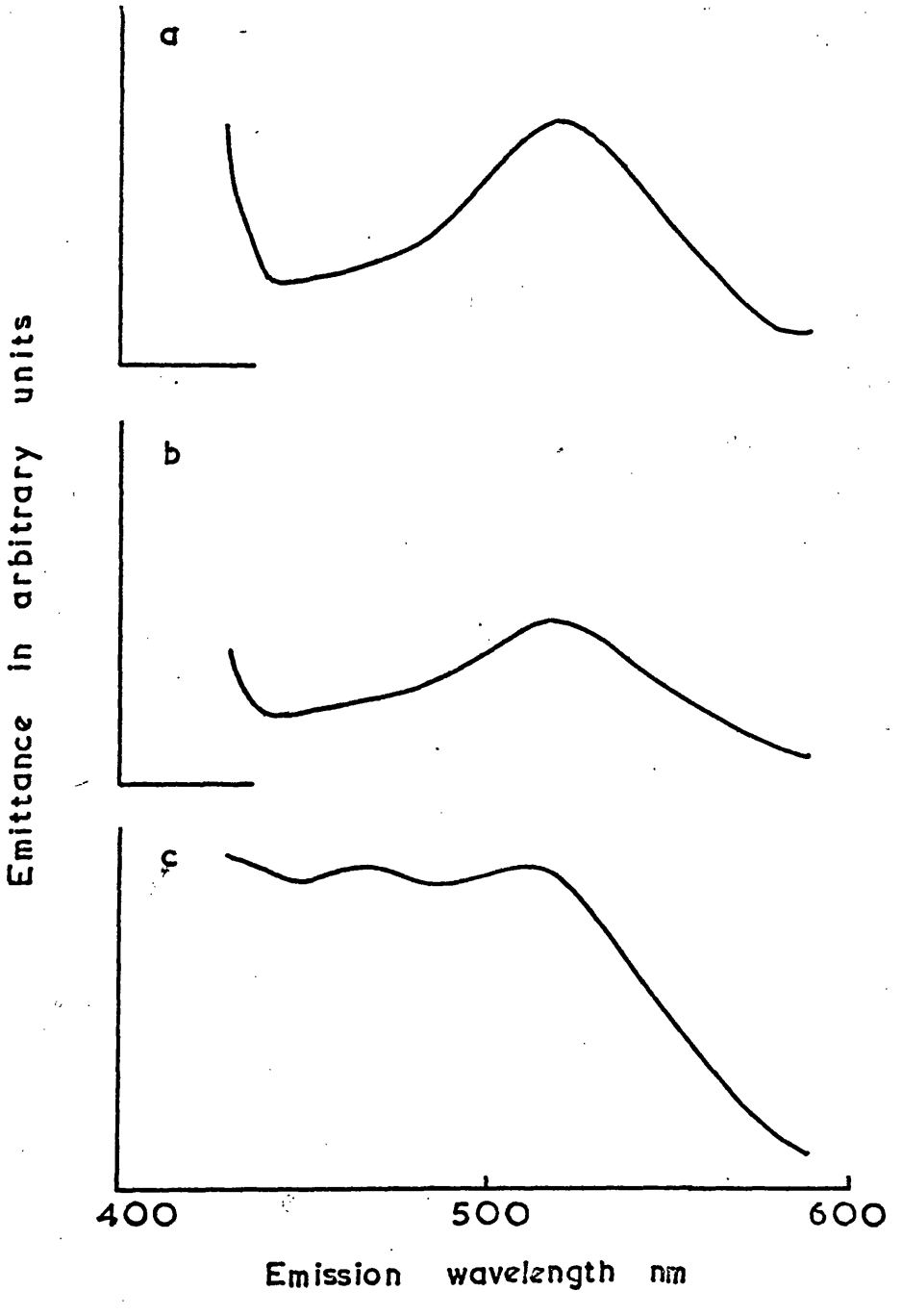

a
b
c
Emittance in arbitrary units
400
500
600
Emission wavelength nm

FIG. 26

A typical growth curve of C.diospyri grown at 25° in Schopfer's medium. Each determination was the average of at least 6 duplicates.

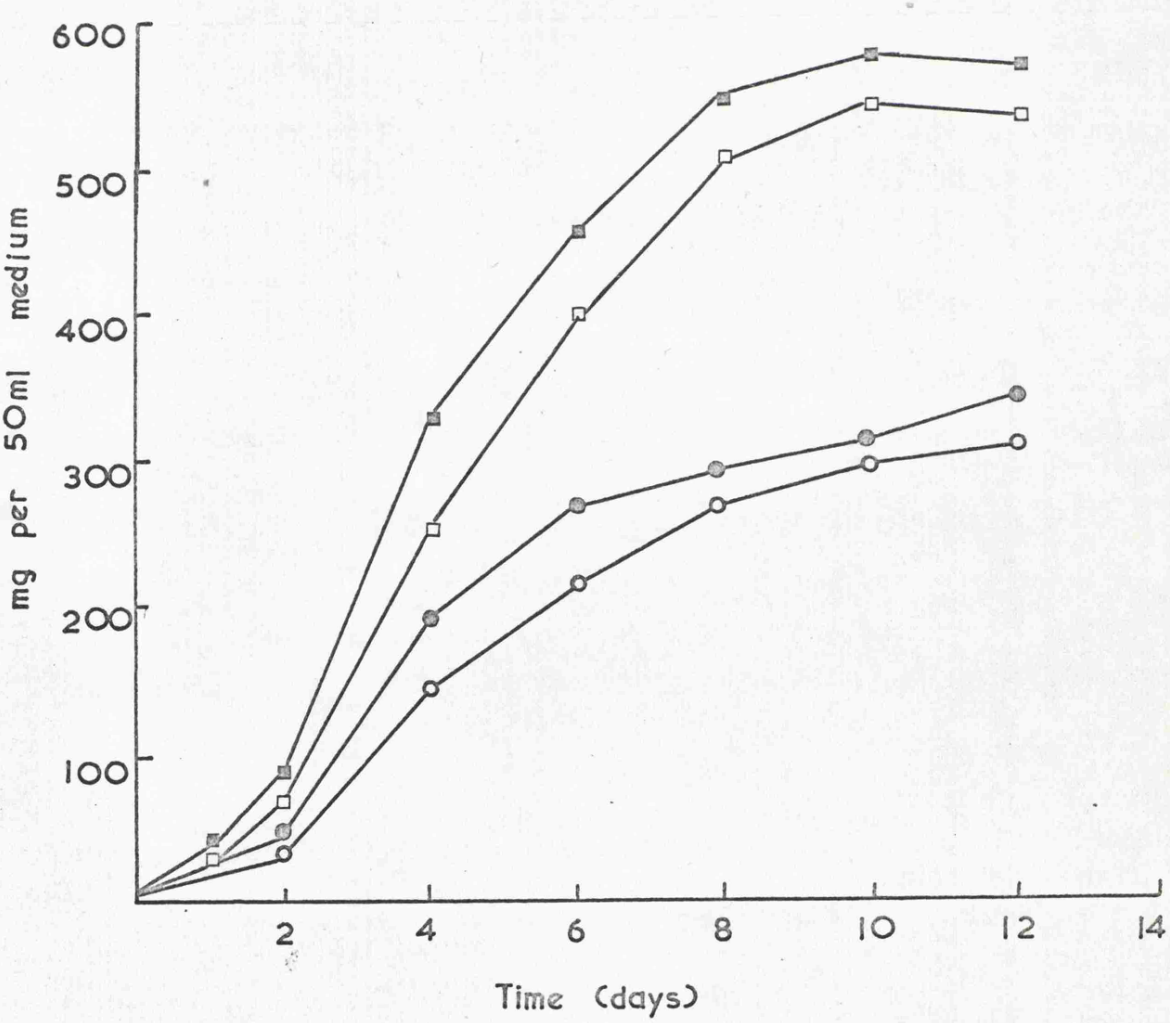
(a) Total dry weight — □—□ Light grown cells

■—■ Dark grown cells

(b) Residual dry weight — ○—○ Light grown cells

●—● Dark grown cells

Light intensity was 2000ft candles


600
500
400
300
200
100
mg per 50ml medium
2
4
6
8
10
12
14
Time (days)

using the butanol + acetic acid + water (4 + 1 + 5 v/v), upper phase solvent system. This allowed good separation of the two compounds, mepacrine having an Rf of 0.83 and fluorescing strongly green, and riboflavin with an Rf of 0.36 and yellow fluorescence. Dark grown culture filtrates contained riboflavin when cells were grown in the presence of 0-1.0mM mepacrine, and, visually at least, there appeared to be very little inhibition of its production induced by mepacrine. In light grown culture filtrates, mepacrine did not appear to protect the photolysis of riboflavin to lumichrome as suggested by Carlile (1962). In addition, mepacrine appeared very stable to illumination, - no other product could be detected chromatographically after exposure of mepacrine to light for 14 days.

(7). The effect of light on the growth of C.diospyri.

Although dry weight determinations have been used in many investigations, their validity as a measure of growth has been questioned by Taber & Siepmann (1965). Mycelium consists of structural or integral cell material as well as storage and metabolic products which cannot be considered as integral parts of the fungus since they may be readily extracted. Also their pattern of accumulation does not necessarily follow that of unextractable parts of the fungus under consideration (Taber, 1964). When dry weight is measured then these compounds are included, but no consideration is given to products synthesised by the cells which have leached into the growth medium. Therefore, total dry weight ought to include products of synthesis plus the mass of all products lost to the medium. It was thought best only to measure integral structures of the cell. Freeze dried tissue was extracted with water for 1 hr. at 100°, the residue weighed and compared to the mass of original freeze dried cells as shown in Fig.26.

Results are in agreement with those of Taber & Siepmann (1965) who found a divergence between the dry weight and residual weight curves of Claviceps purpurea, indicating that the total dry weight curve was the

sum of several curves including that for residual mycelium. Although every precaution was taken to standardise inocula, total dry weight produced did show variation from experiment to experiment. Replicates within each experiment showed good agreement (+ 5%). In most growth experiments, dry weights of illuminated cultures on Schopfer's medium were slightly lower than those grown in the dark. Attempts to define growth in terms of 'residual' mass showed similar differences of about 10% between light and dark grown cultures (Fig.26). Cells grown in submerged culture in Malt broth and Czapek dox broth (Table XIIIa) showed very little difference in total dry weight production when in the presence and absence of light.

TABLE XIIIa.

Total biomass and residual biomass of C.diospyri grown in submerged culture in Malt broth and Czapek dox broth at 25°. Each value quoted is the mean of six duplicates.

Culture Medium		Age of Cells	Total Mass	Residual Mass
			mg/50ml medium	
Malt	Light	6	320 ± 24	152
	Dark	6	325 ± 28	158
C.Dox	Light	6	249 ± 22	126
	Dark	6	258 ± 27	130

TABLE XIIIb

% hot water extractable material of cells grown in Schopfer's medium. Initial amount of fungus was 100mgm. dry weight.

Age of Cells	Light	Dark	Light	Dark
	Residual dry weight		% Total Carbohydrate extracted	
4	59	60	9.7	10.7
6	54	55	11.2	11.4
8	52	53	10.4	10.1
10	54	54	8.6	10.0
12	62	63	7.3	8.2

Table XIIIb. shows that the percentage of water extractable material from both light and dark grown cultures was the same, suggesting that the biosynthetic capacity for both was similar. Hot water extractable carbohydrate values also showed close agreement. For most analytical experiments the fungal material was harvested after 6 days growth. Although maximal growth had not been obtained, residual growth had nearly been reached. Harvesting at this cell 'age' seemed to be a sound practical approach to comparative experiments performed in this investigation.

Effect of Mepacrine on the growth of C.diospyri.

Methods of elucidating possible photoreceptors have quite often consisted of observing effects of specific inhibitors to the compounds in question of the photoresponse. Mepacrine has been used in this manner by Carlile (1962) who, on the basis of its effect on Phycomyces inferred a flavoprotein difference between light and dark grown cultures. When added to Schopfer's medium in the same range of concentrations (0-1.0mM) as used by Carlile, light and dark grown cultures of C.diospyri were neither preferentially inhibited (Fig.27) in total biomass production.

Light and dark grown cultures of C.diospyri in Schopfer's medium containing diphenylamine (10^{-4}M - 10^{-5}M) did appear to show differences in final growth yield at the higher concentrations of inhibitor employed (Fig.27).

(8) Chemical Composition of cells of C.diospyri.

Cells grown in Schopfer's medium under conditions of light and dark for 6 days were analysed as a preliminary attempt to find out if there were any differences between them. The results are shown in Table XIV. From this data it would appear that light did not significantly alter any of the cell parameters measured. Attempts to detect DNA with diphenylamine in extracts from the sample size used were unsuccessful.

FIG. 27

The effect of metabolic inhibitors upon total dry weight production by light and dark grown cells of C.diospyri in Schopfer's medium. Cells harvested after 6 days at 25°.

(a) Mepacrine

(b) diphenylamine

O—O : light grown cells

●—● : dark grown cells

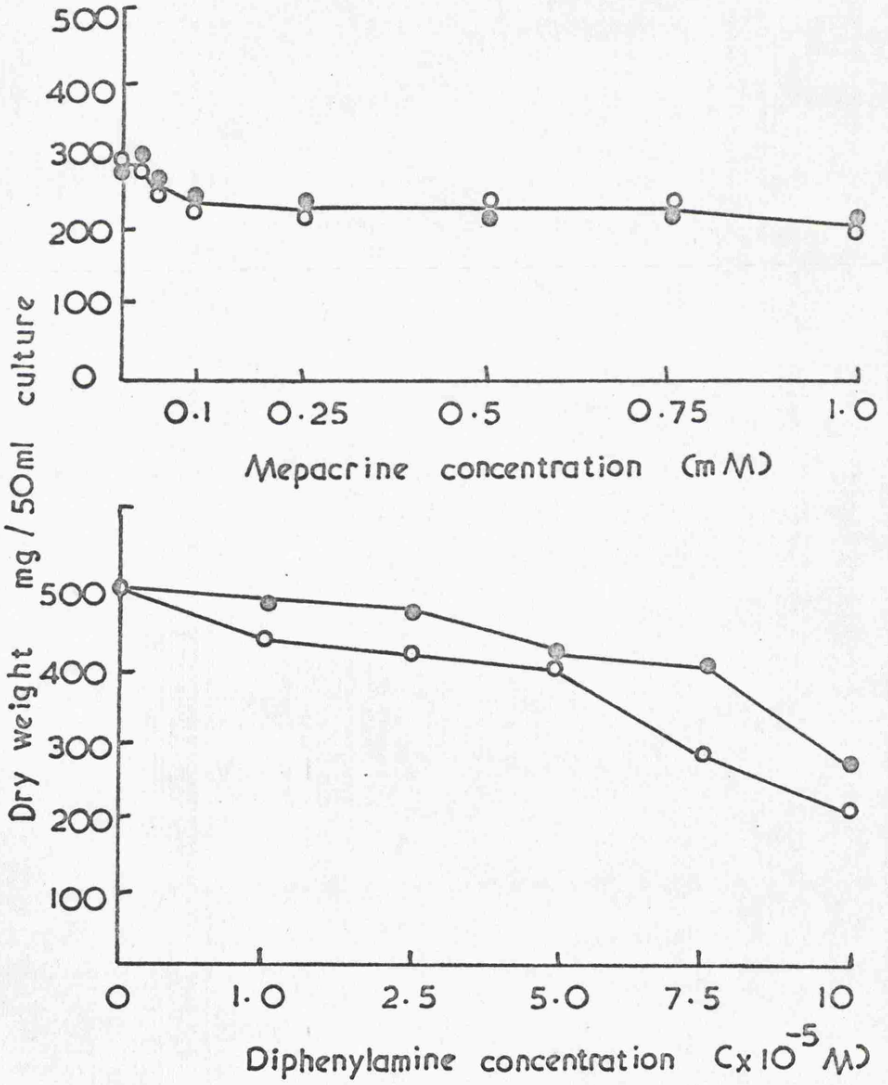

500
400
300
200
100
0
Dry weight mg / 50ml culture
0·1
0·25
0·5
0·75
1·0
Mepacrine concentration (m M)
500
400
300
200
100
0
1·0
2·5
5·0
7·5
10
Diphenylamine concentration (x 10⁻⁵ M)

TABLE XIV.

Chemical composition of C.diospyri grown for 6 days at 25° in Schopfer's medium. Results expressed as a percentage of total dry weight.

Cell Constituent	Light Grown Cells	Dark Grown Cells
Total Lipid	9.4 ± 0.70	9.8 ± 0.90
Total N_2	5.2 ± 0.35	4.9 ± 0.25
TCA sol. N_2	0.95 ± 0.14	1.04 ± 0.17
Total Phosphorus	1.38 ± 0.11	1.50 ± 0.15
TCA sol.Phosphorus	0.42 ± 0.04	0.44 ± 0.06
RNA	0.38 ± 0.04	0.40 ± 0.05
DNA	None detected	none detected

(9) Sterol content of C.diospyri.

Both sterols and carotenoids are formed from isoprenoid 5-C units, and their biosynthetic pathways share a number of common steps up to the formation of farnesyl pyrophosphate. It therefore seemed pertinent to examine light and dark grown cells for their sterol content. Values were estimated from a standard curve prepared with ergosterol. It may be seen from Table XV that light did not appreciably effect the sterol content of cells grown in Schopfer's medium. The values obtained appear to be in good agreement with those quoted by Appleton et al (1956) for C.acremonium of 0.68% (w/w).

TABLE XV.

Sterol content of cells grown in Schopfer's medium for 6 days at 25°.

Culture conditions	Sterol content % dry weight
Light	0.66 ± 0.09
Dark	0.63 ± 0.07

(10) Pyrollysis of Cells.

The objective of this experiment was to obtain an overall picture of the metabolism of the fungus which might help in deciding if there were significant changes induced by the light stimulus. Therefore freeze dried cells of C.diospyri were pyrolised and the patterns obtained are shown in Fig.28. These profiles even though complex are qualitatively identical in both major and minor peaks. Quantitatively, although different amounts of fungal tissue were used in this experiment, the relative peak heights of many components from light and dark grown cells of C.diospyri were markedly different, suggesting possible differences in chemical composition between them. However, the main disadvantage using a technique such as pyrolysis is that there is no method for determining the identity of the individual components easily.

(11). Amino Acid pools from cells grown in the light and dark.

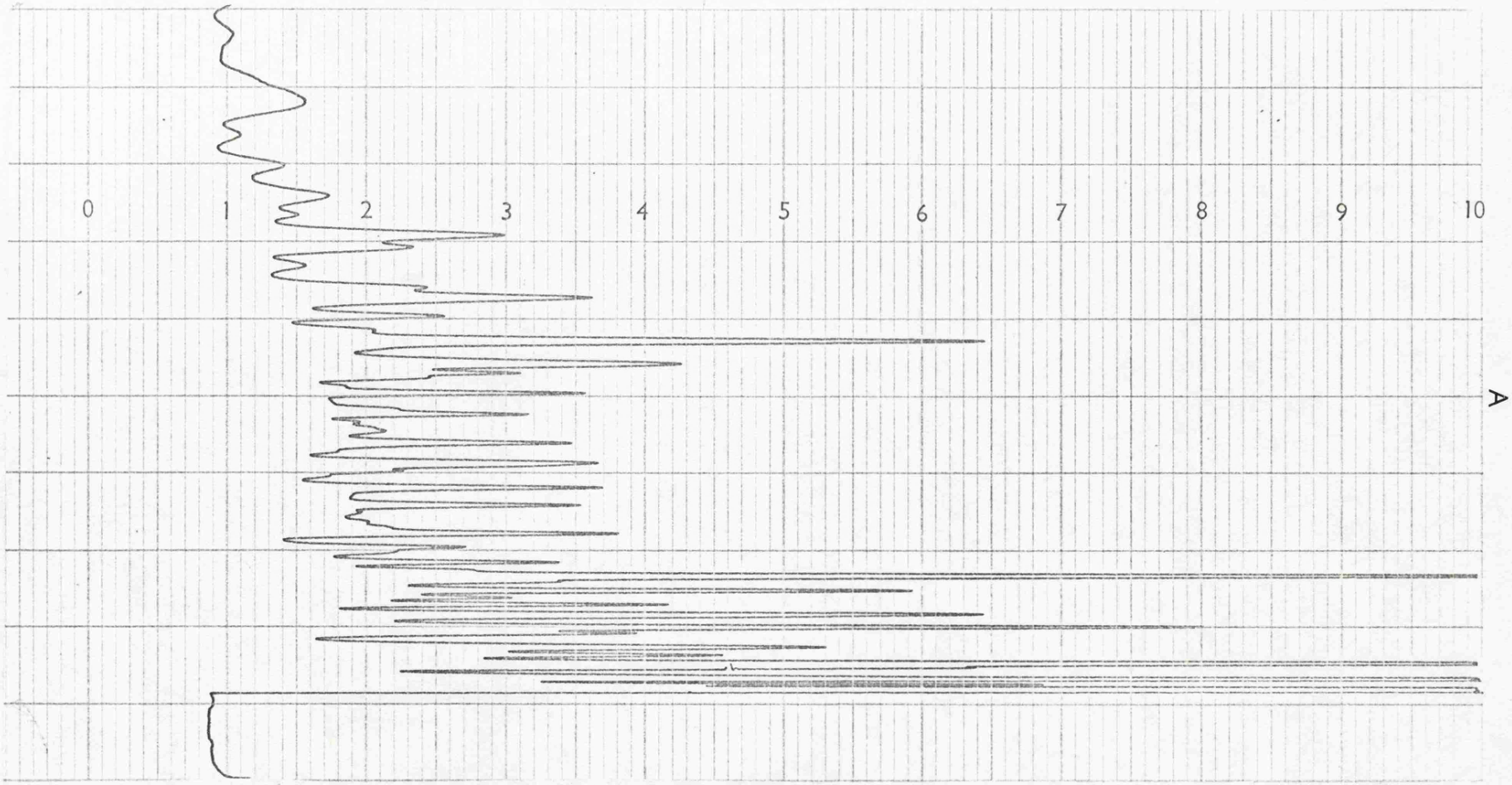
The free amino acid composition of cells grown in Schopfer's and Czapek dox medium is shown in Plate VII. Qualitatively, the pools were the same from both light and dark grown cells of C.diospyri, each with 14 well defined components. Tentative identification based on distance travelled and colour reaction with ninhydrin showed that alanine was present in the highest amounts with lysine, isoleucine or leucine, methionine and arginine in less amounts. Proline and hydroxyproline were not detected in the amino acid pool of cells grown in these two media, but cells grown in Malt broth did appear to contain proline in both light and dark culture. Quantitatively too, the relative amounts of amino acids in light and dark grown cells appeared to be very similar visibly, and pools fro m cells grown on Czapek dox broth were larger than those from cells on Schopfer's medium. Noticed on all chromatograms was one unidentified component running farther

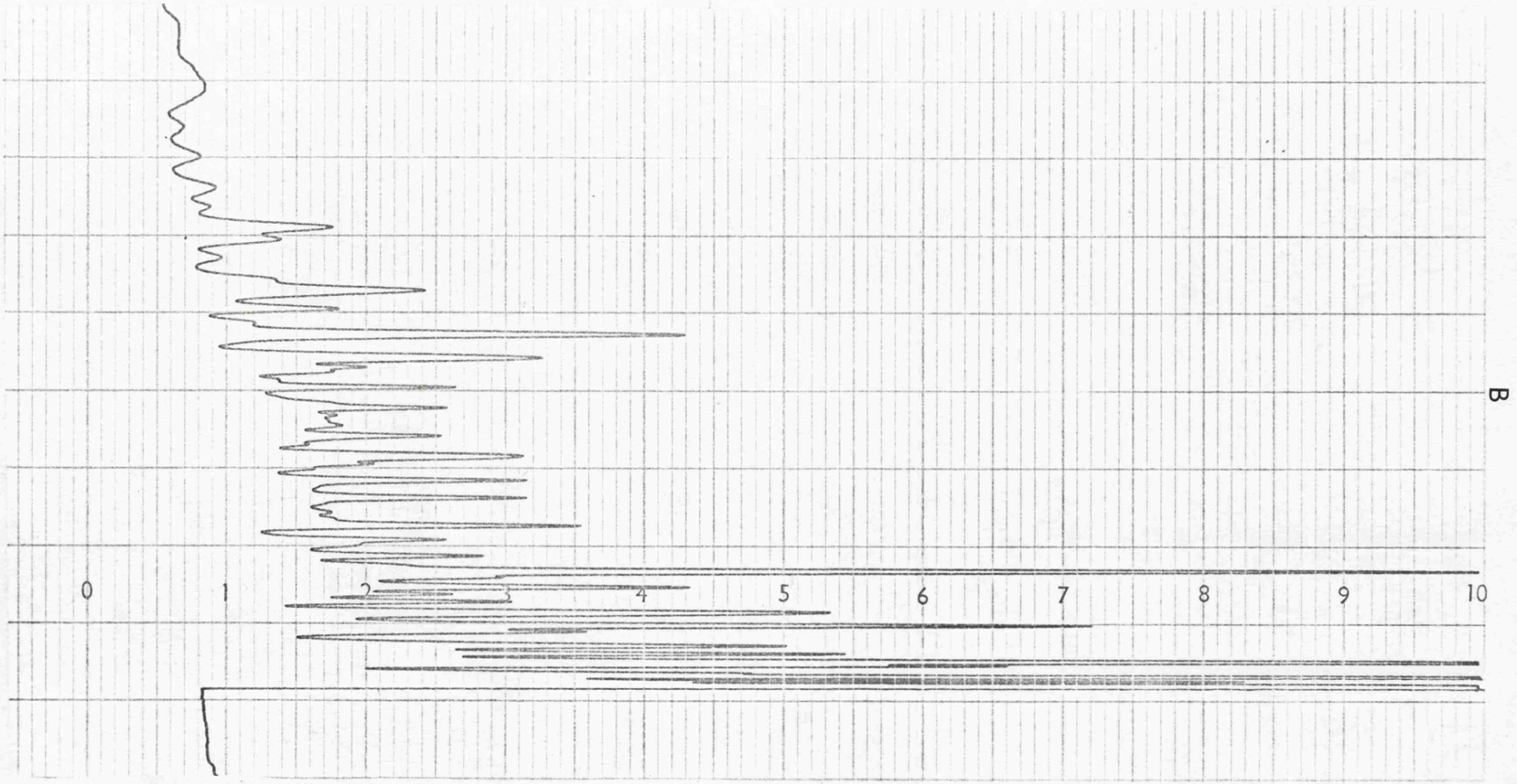
FIG. 28

Pyrollysis of 6 day old light and dark grown cells of C.diospyri from Schopfer's medium. Method as described in the text.

A: light grown cells

B: dark grown cells.


0
1
2
3
4
5
6
7
8
9
10
A


B
0
1
2
3
4
5
6
7
8
9
10

than leucine or isoleucine with an Rf of 0.78, appearing later than the other separated components after colour development. The qualitative pattern of the pool was stable. No major changes were detected over a period of 4 to 10 days in cells grown in Schopfer's medium.

Analysis of culture filtrates of cells grown on Schopfer's medium revealed little ninhydrin positive material on separated chromatograms from 6 day old cultures. Results were the same for both cells grown in the light and the dark. Very poor separation was achieved, although most of the extracellular glucan was removed with ethanol. These results are in partial accord with those of Tegtmeier & Pappelis (1966), who reported that light did not alter significantly the composition of the mycelial amino acid pools in Diplodia zeae. However, the amino acid content of culture media increased greatly in light grown cultures. From results obtained, there was no evidence to suggest the possible light mediated leakage of amino acids from cells of C.diospyri.

(12). Organic Acid content of C.diospyri.

Light and dark grown cells of C.diospyri were analysed for their organic acid content. A chromatographic separation of organic acids is shown in Plate VIII. Identification from authentic standards were, in order of decreasing mobility, fumarate, an unknown compound with an Rf of 0.79, succinate, malate and isocitrate or citrate. However there was no qualitative difference between extracts from light and dark grown cultures, and no obvious quantitative differences. The same organic acid composition was found in cells harvested after 4, 6, 8 and 10 days growth in Schopfer's medium.

Extracts from cells grown on C.dox broth were also found to contain the organic acids reported above as well as an extra unidentified compound visible in extracts from both light and dark grown cells. The very slow moving acidic material present on all chromatograms was most

Plate VII

Separated Amino Acid pools of C. diospyri. Method as described in the text.

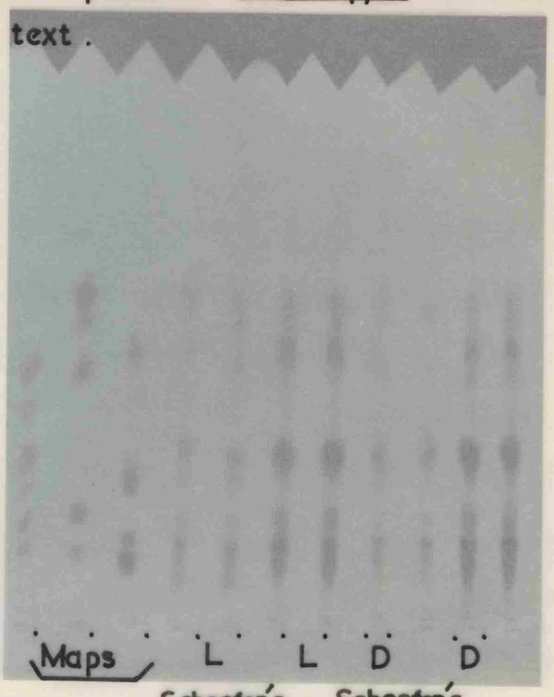
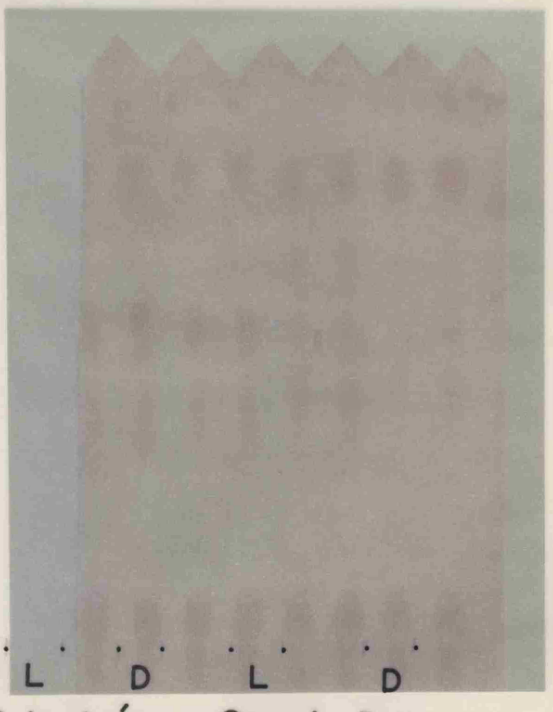
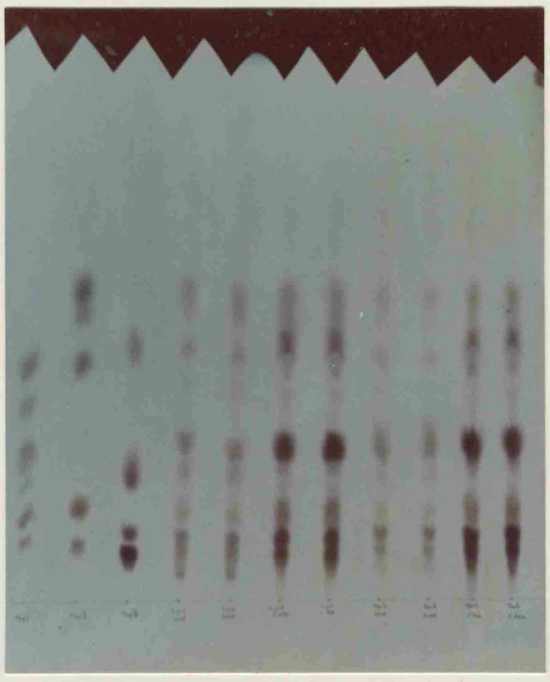
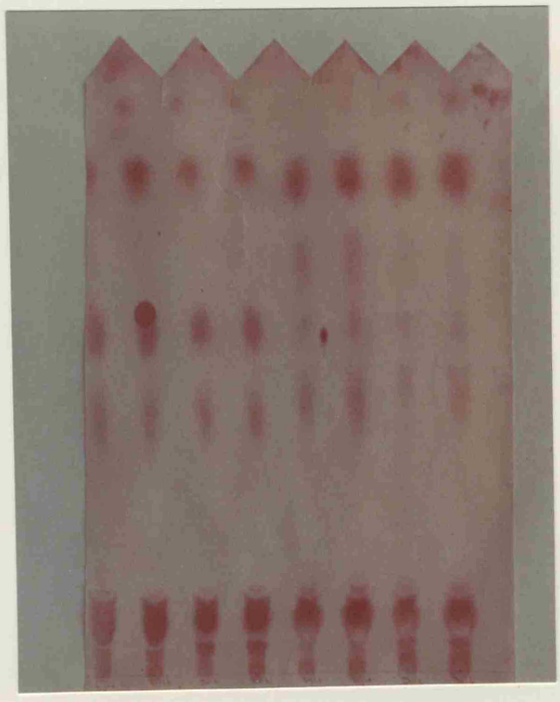


Plate VIII

Organic acids of light and dark grown cells of C. diospyri.







probably due to interfering anions (Cl^- etc.,), since any anion which replaces H^+ in the indicator would give a positive colour. It was found that in passage through the ion exchange columns recovery of standard organic acids was good, except in the case of αoxoglutarate, of those tried. This acid was totally lost after passage of the standard through the Amberlite IRA 400 (CO_3^{---}), but not appreciably on passage through Dowex 50 (H^+).

Chromatographic analyses of culture filtrates of both light and dark cells grown in Schopfer's and C.dox medium revealed one major component together with traces of other acids tentatively identified as citrate and succinate. This major compound had, like the unidentified compound in extracts from cells, an Rf of 0.79 in the solvent system used, and from this position was thought to be either glutaric or itaconic acid. By running chromatograms and spraying them with 2% (w/v) aqueous potassium permanganate, washing them under hot water, the unknown compound appeared as a brown spot on a white background. This was taken as evidence for the acid being itaconate, since the presence of a double bond would produce this colour reaction whereas glutaric acid would give no colour (Hulme, 1961). Therefore cells, as well as culture filtrates appeared to contain appreciable amounts of itaconate, although light did not appear to alter quantitatively its production by C.diospyri.

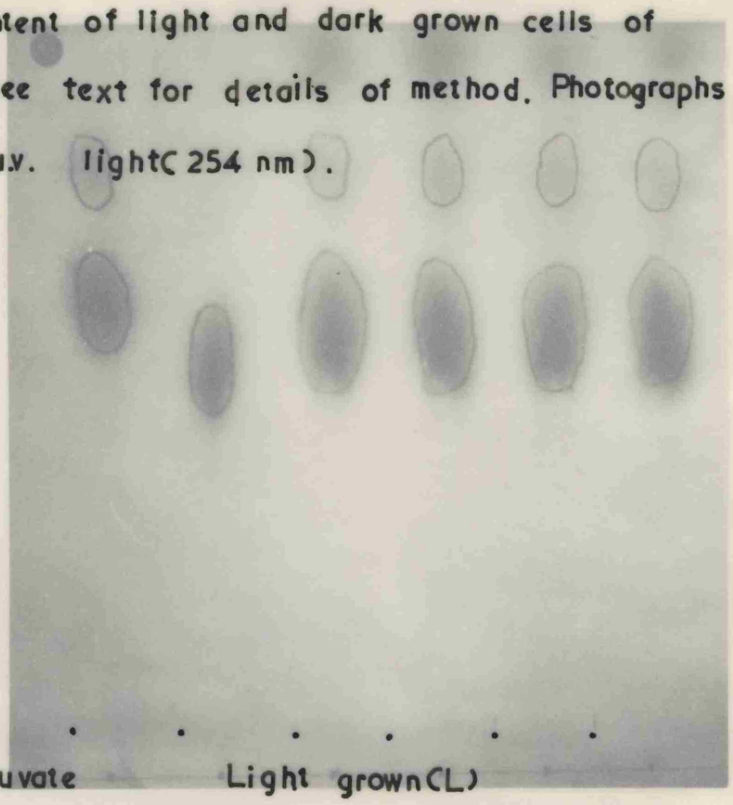
(13). Keto Acid content of C.diospyri.

As important intermediate metabolites, the keto acid composition of cells grown in light and darkness was investigated. Their presence and identification was determined by the method described previously. The Plate IXa, shows separation of DNP derivatives in the solvent system, (a) butan-1-ol + ethanol + water (4 + 1 + 5 v/v). No separation was possible between the first isomer of the pyruvate derivative and that of the αoxoglutarate derivative. A much improved separation was achieved using the solvent system (b) of butan-1-ol + ethanol + 0.5N NH_4OH

Plate IX

Keto acid content of light and dark grown cells of C. diospyri. See text for details of method. Photographs taken under u.v. light (254 nm).

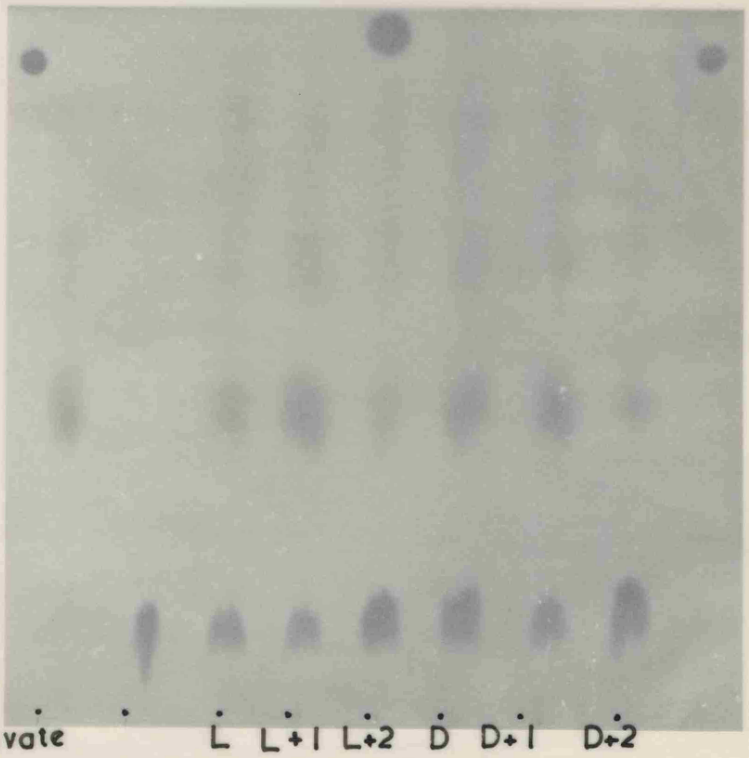
Plate IX (a)



Pyruvate Light grown (L)

α-Oxoglutarate Dark grown (D)

Plate IX (b)



Pyruvate L L+1 L+2 D D+1 D+2

(1)

α-Oxoglutarate

(2)

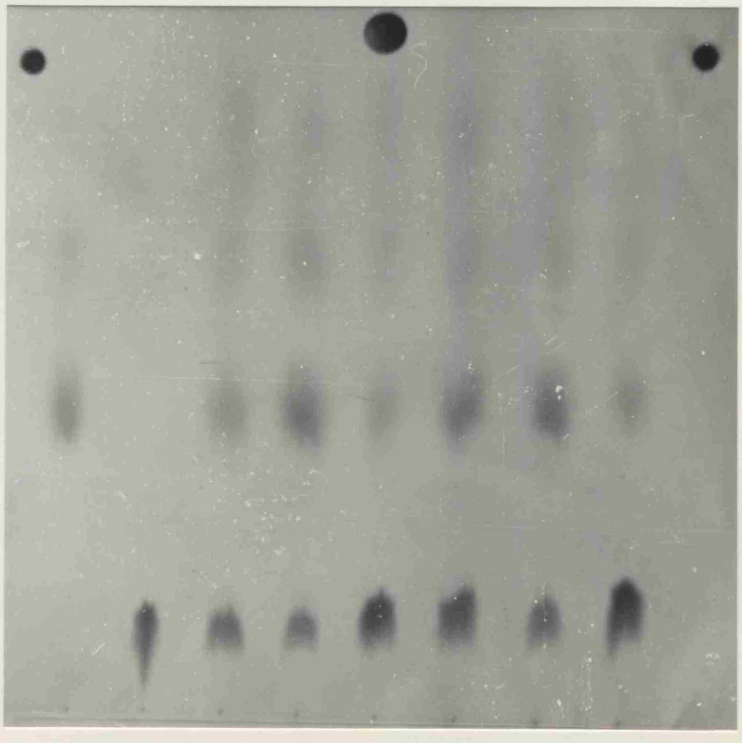
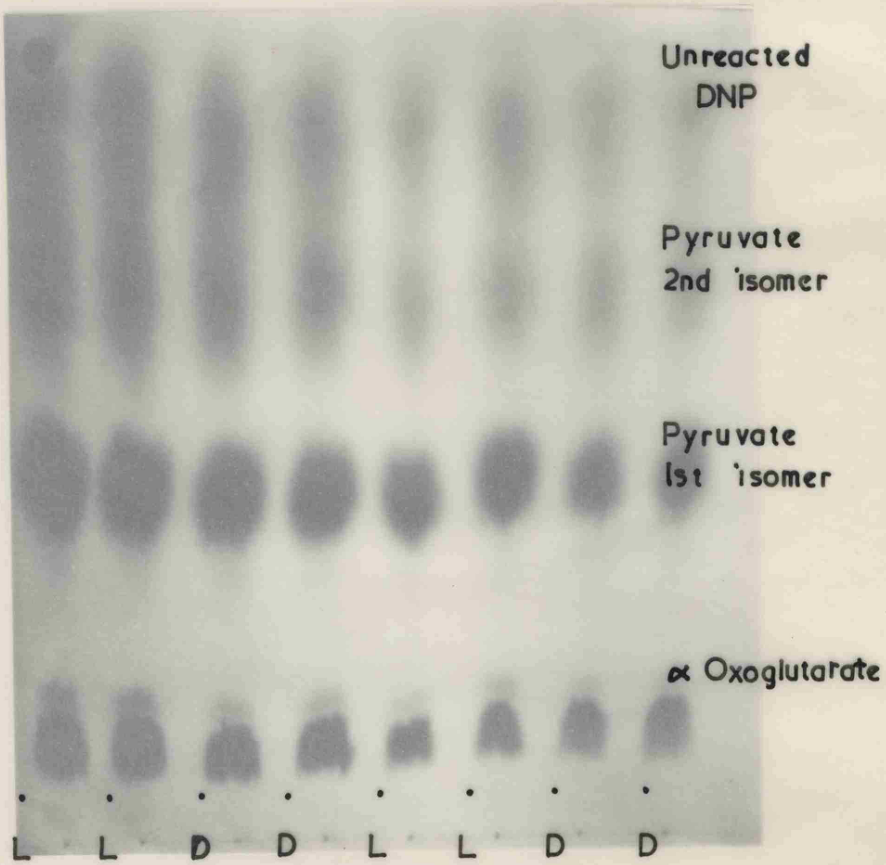
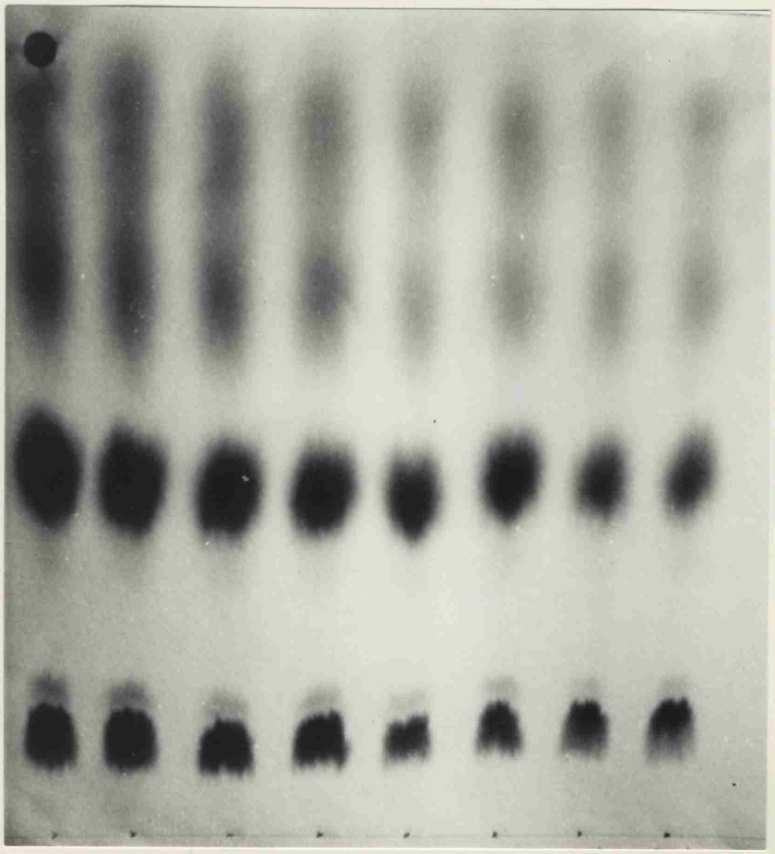


Plate IX (c)

Keto acids for culture filtrates of C. diospyri.





(7 + 1 + 2 v/v) where the presence of ammonium ions slowed down the dicarboxylic acid derivative as shown in Plant IXb. Cells of C.diospyri therefore contained only pyruvate and α oxoglutarate in any detectable quantities. Co-chromatography with authentic samples of DNP derivatives was used as confirmatory evidence of their identification. From these chromatographs, there appeared to be no qualitative difference in keto acid content of light and dark grown cells of C.diospyri. The yellow spots running with or near the solvent front on the chromatographs were due to unreacted DNPH in the samples. Extracts from cells grown in C.dox medium also revealed the presence of only pyruvate and α oxoglutarate. Rf values obtained in solvent system (a) were; α oxoglutarate (0.55) pyruvate (0.63) and pyruvate isomer (0.80) and in solvent system (b) were α oxoglutarate (0.13), pyruvate (0.47) and pyruvate isomer (0.70).

Culture filtrates were also analysed in the same manner (Plate IXc). In those from light and dark grown cells both pyruvate and α oxoglutarate were detected, as well as an extra unidentified component with an Rf in solvent system (b) of 0.22.

The keto acid content of cells grown in Schopfer's medium over the growth period of 12 days is shown in Table XVI. From this data there appears to be approximately twice as much α oxoglutarate as pyruvate in cells of C.diospyri. However, the effect of light upon their composition appeared to be negligible. Similar values were obtained when cells grown on Czapek dox medium were analysed.

FIG. 29.

Keto acid production by C.diospyri grown in Schopfer's medium at 25°.

 : Pyruvate - light grown culture filtrates

 : Pyruvate - dark grown culture filtrates

 : ∝oxoglutarate - light grown culture filtrates

 : ∝oxoglutarate - dark grown culture filtrates

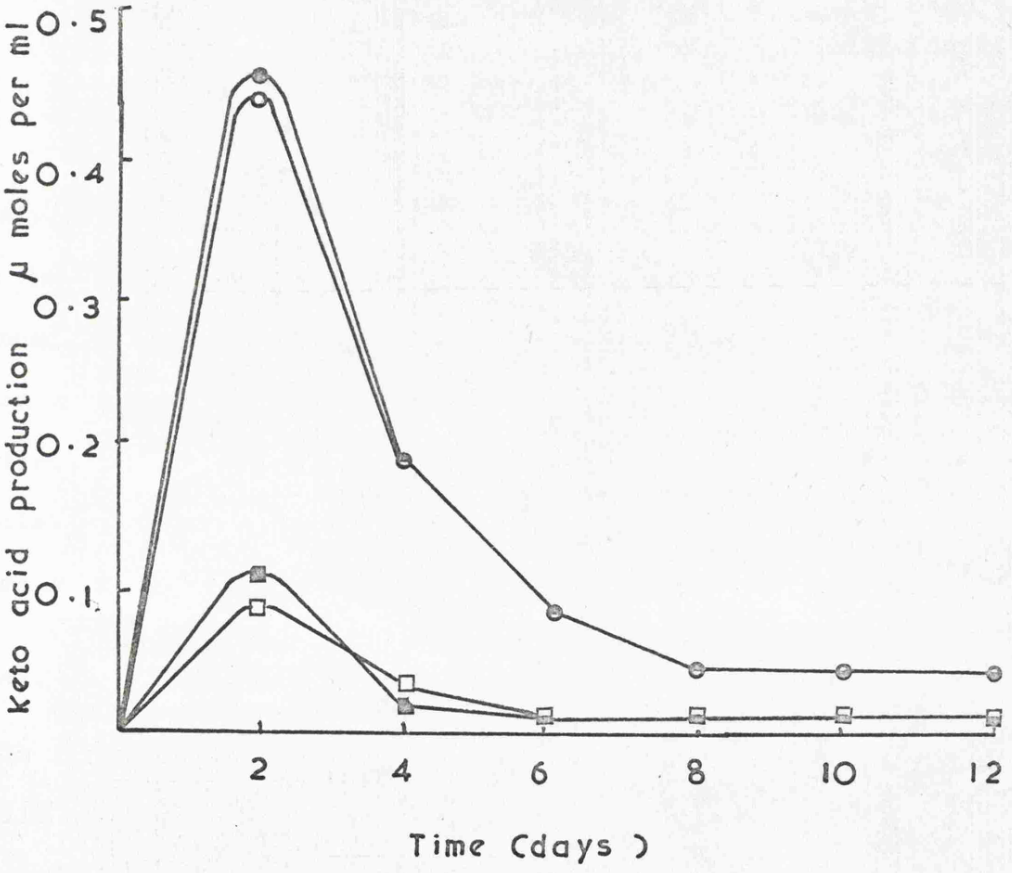

Keto acid production μ moles per ml
0·5
0·4
0·3
0·2
0·1
2
4
6
8
10
12
Time (days)

TABLE XVI

Keto acid content of cells grown in Schopfer's medium. Mean of triplicates from three determinations. Results expressed as μmoles/g. dry weight of C.diospyri.

Age of cells (days)	Pyruvate		α oxoglutarate	
	Light	Dark	Light	Dark
4	4.16	4.24	9.92	10.11
6	4.40	4.12	9.45	10.02
8	4.26	4.09	10.21	10.44
10	4.00	3.74	9.47	9.85
12	3.57	3.60	9.81	9.82

Analysis of filtrates of cells grown in Schopfer's medium, revealed that in the early stages of growth after 2 days, pyruvate accumulated in large amounts roughly four times as much as α oxoglutarate. In cultures older than 2 days, these acids were found to disappear from the medium. Filtrates from both light and dark grown cultures followed the identical pattern, so it would appear that the keto acids were reutilised at the same rate in both (Fig.29).

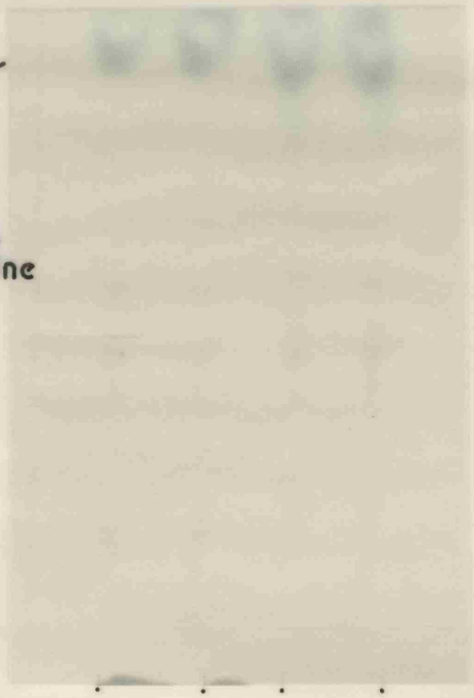
(14). Lipid analysis of C.diospyri.

Thin layer chromatography of phospholipids from light and dark grown cells of C.diospyri was performed as described in the Materials and Methods section. Analysis revealed the presence of phosphatidyl ethanolamine, phosphatidyl glycerol, phosphatidyl choline and phosphatidyl inositol, identified tentatively from their positions in relation to known standards. Qualitatively there was no difference between light and dark grown cells.

Total lipid analysis revealed extracts to contain neutral lipids and pigments (not shown in Plate X), sterol fraction, four components which have not been identified, and the slower running phospholipids already mentioned. It is of interest that the compound running just behind the

Plate X

Thin layer chromatography of a Total lipid extract of cells of C.diospyri. Method of separation and identification where possible described in the text.



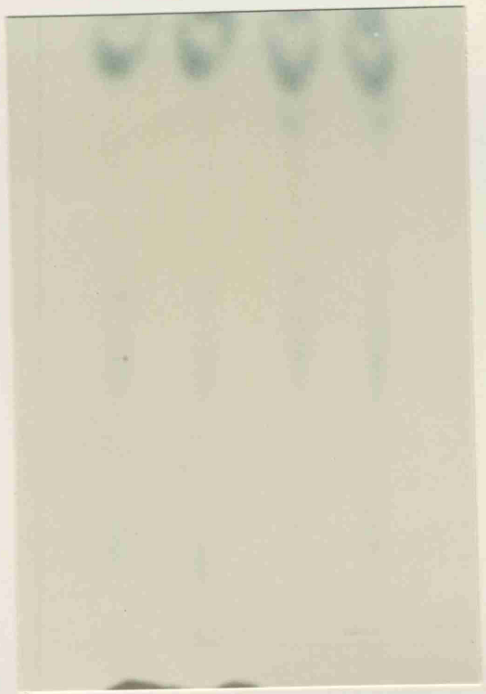
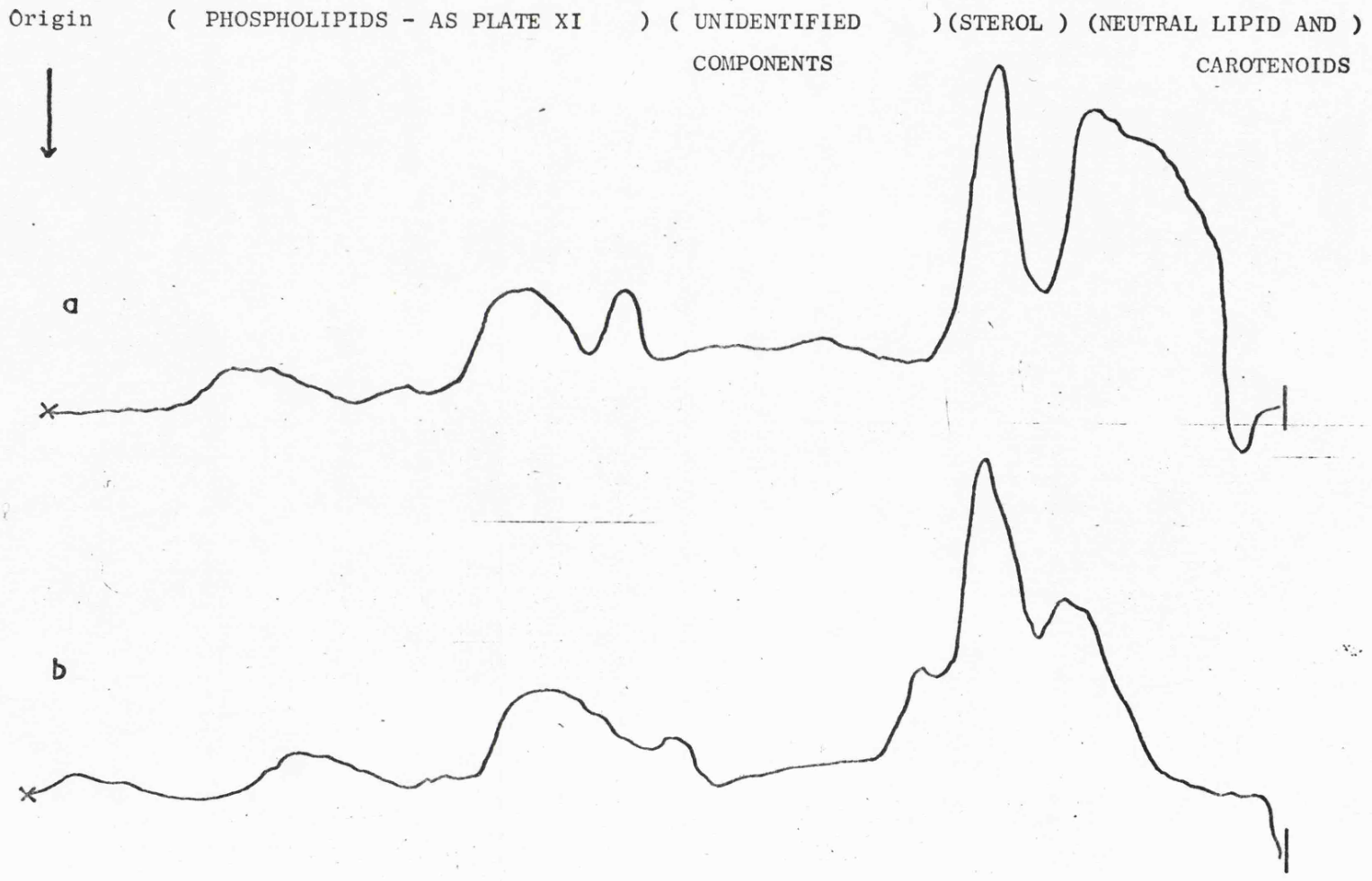


FIG. 30.

Chromoscan traces of chromatograms of separated lipids of light and dark grown cells of C.diospyri. Analyses, described in the text, was carried out on 6 day old cells grown in Schopfer's medium at 25°.

(a) Light grown cells

(b) Dark grown cells.


Origin
(PHOSPHOLIPIDS - AS PLATE XI)
(UNIDENTIFIED COMPONENTS)
(STEROL)
(NEUTRAL LIPID AND CAROTENOIDS)
a
b

sterols (Fig.30) and easily detectable in dark grown cells of C.diospyri was present in much smaller amounts, hardly detectable visibly in developed chromatograms of extracts from light grown cells. Unfortunately, this compound was not identified, but further investigation in this area is obviously needed.

(15). Electrophoresis of soluble proteins from C.diospyri.

Electrophoretic patterns for proteins and isoenzymes from light and dark grown cultures of C.diospyri in Schopfer's medium are shown in Figs 31-34. Protein concentrations of extracts used varied between 2.8 and 5.2 mg/ml, and under the conditions of electrophoresis 20 protein bands were regularly detected on gels. Experimental variation appeared to be quite small, since identical protein patterns were obtained from batches of cells grown 6 months apart. The chromoscan traces shown that light did not have any effect qualitatively on protein profiles from cells of C.diospyri, although there did appear to be differences in the relative band intensities between them. Shift in baseline due to un-uniform destaining of the gels tended to confuse the gel scan patterns and it is suggested that clearer results would be obtained by scanning unstained gels at 280nm for protein, when this problem would be eliminated, if all proteins showed equal absorption at 280nm.

Gels stained for esterase activity with butyrate ester as the substrate were faint replicas of the acetate ester patterns, most probably due to lower activity of esterases to the longer chain fatty acids. In all gels of separated esterases, seven bands were detectable. However, once again light did not alter the distribution of them. Alkaline phosphatases on all gels showed one major band corresponding to that of the fastest moving acid phosphatase isoenzyme, and very faint bands in the same position as the two slower moving isoenzymes in the acid phosphatase system. Thus it would suggest that these phosphatases, in vitro at least, have a wide pH tolerance for activity. Both acid and alkaline phosphatases gave identical patterns in both light and dark

FIG. 31

Densitometer traces of Polyacrylamide gels of soluble proteins from 6 day old cells of C.diospyri grown in the light and dark. Method of electrophoresis and staining as described in the text.

Conditions of scanning on Chromoscan.

Cam; 1:3

Wedge: 5-077A

Slit; variable

Filter; 620

(a) light grown cell extract

(b) dark grown cell extract

Schopfer's medium.

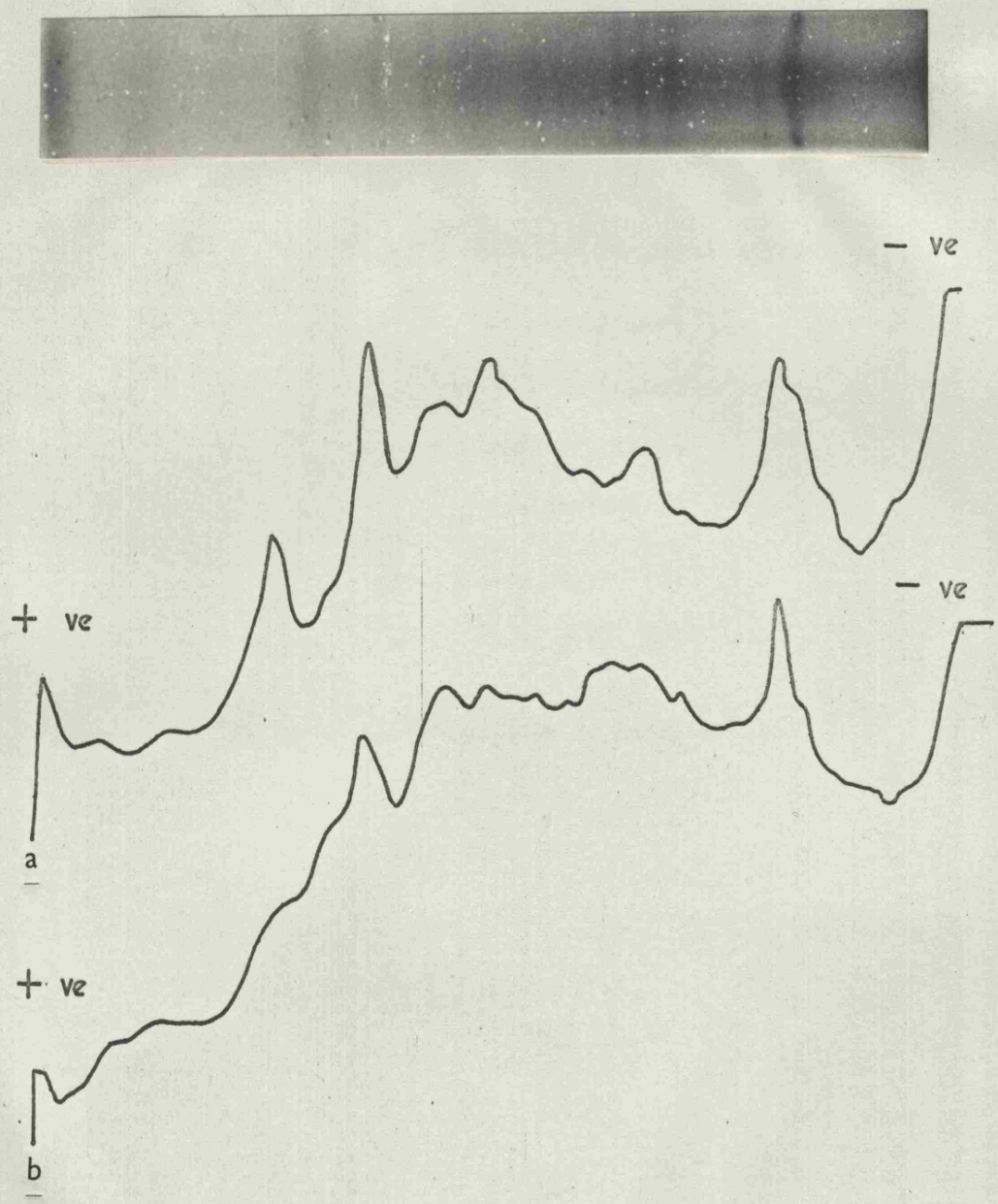

− ve
+ ve
− ve
a
+ ve
b

FIG. 32.

Densitometer traces of Esterase (acetate) Isoenzymes from light and dark grown cells of C.diospyri. Method of histochemical staining used was as described in the text.

Conditions of scanning on Chromoscan.

Cam; 1:3

Wedge; 5-077C

Slit; variable

Filter; none

(a) light grown cells

(b) dark grown cells

Cells grown in Schopfer's medium at 25^{o} for 6 days.

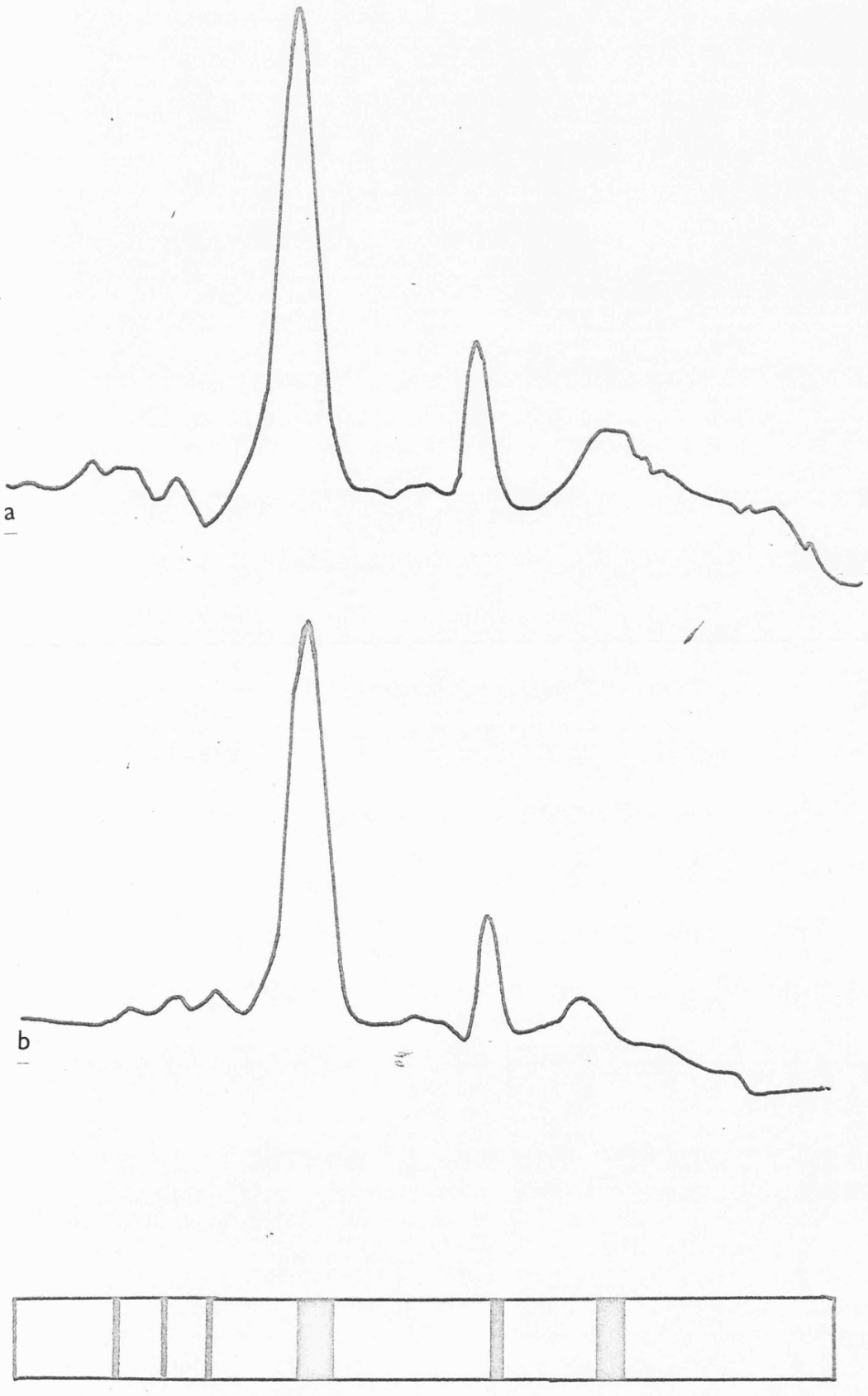

a
b

FIG. 33.

Densitometer traces of acid phosphatase isoenzymes from light and dark grown cells of C.diospyri. Method of electrophoresis and histochemical staining as described in the text.

Conditions of scanning on the Chromoscan.

Cam: 1:3
Wedge; 5.077A
Slit; variable
Filter; 420

(a) light grown cells

(b) dark grown cells.

Cells grown in Schopfer's medium at 25° for 6 days.

FIG. 34.

Densitometer traces of catalase isoenzymes from light and dark grown cells of C.diospyri. Method of electrophoresis and histochemical staining as described in the text.

Conditions of scanning on the Chromoscan.

Cam; 1:3
Wedge; 5-077A
Slit; variable
Filter; none

(a) light grown cells
(b) dark grown cells

Cells grown in Schopfer's medium at 25° for 6 days.

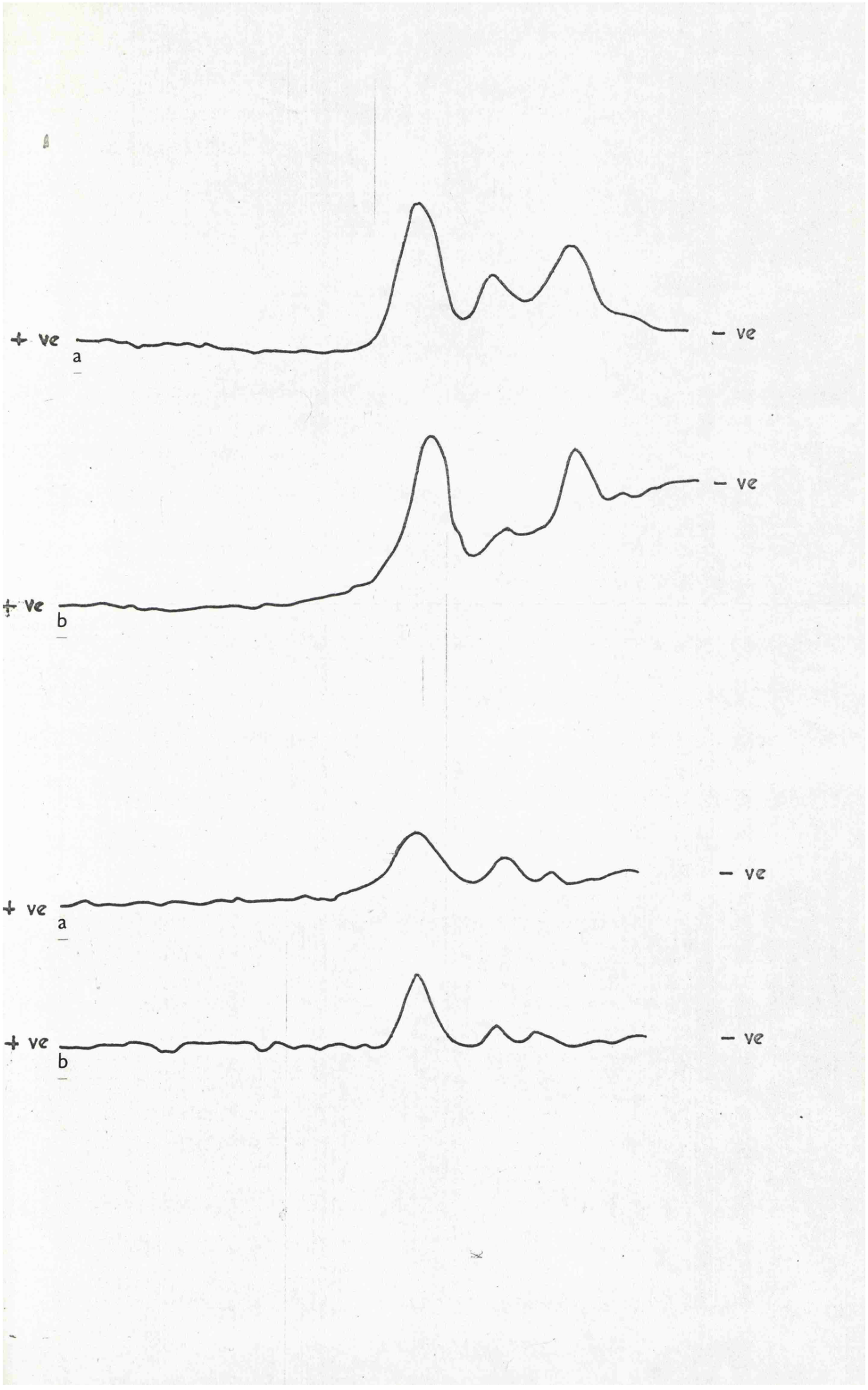

+ ve
a
- ve
- ve
+ ve
b
- ve
+ ve
a
+ ve
b
- ve

FIG. 35.

Densitometer traces of soluble proteins from light and dark grown cells of C.diospyri separated on Polyacrylamide gels.

Conditions of scanning as described in Fig.31.

Cells grown in Malt Broth at 25^{o} for 6 days.

(a) light grown cells

(b) dark grown cells.

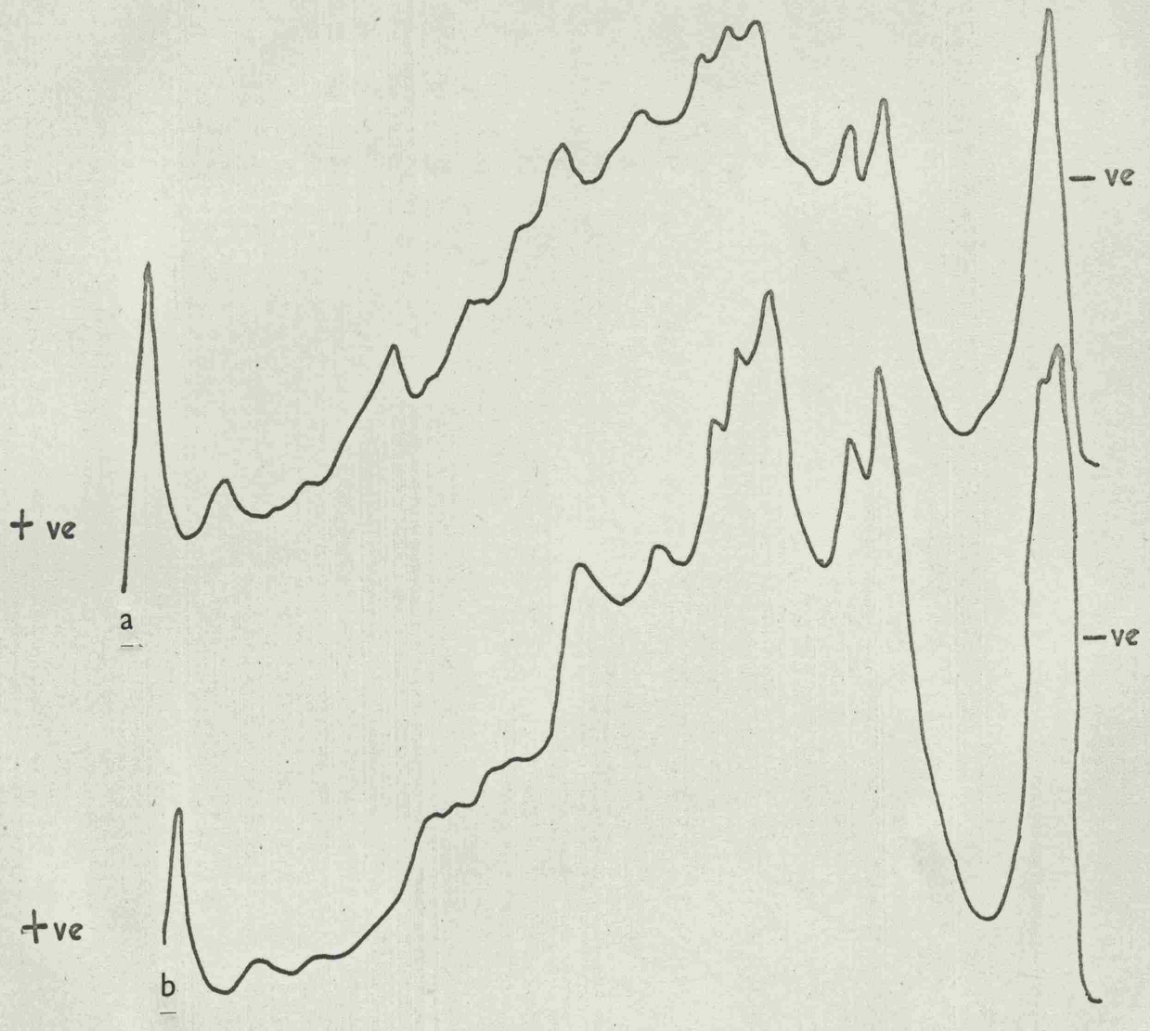

+ ve
a
− ve
+ ve
b
− ve

FIG. 36.

Densitometer traces of Esterase (acetate) isoenzymes from light and dark grown cells of C.diospyri.

Conditions of scanning as described in Fig.32.

(a) light grown cells

(b) dark grown cells

Cells grown in Malt Broth at 25^{o} for 6 days.

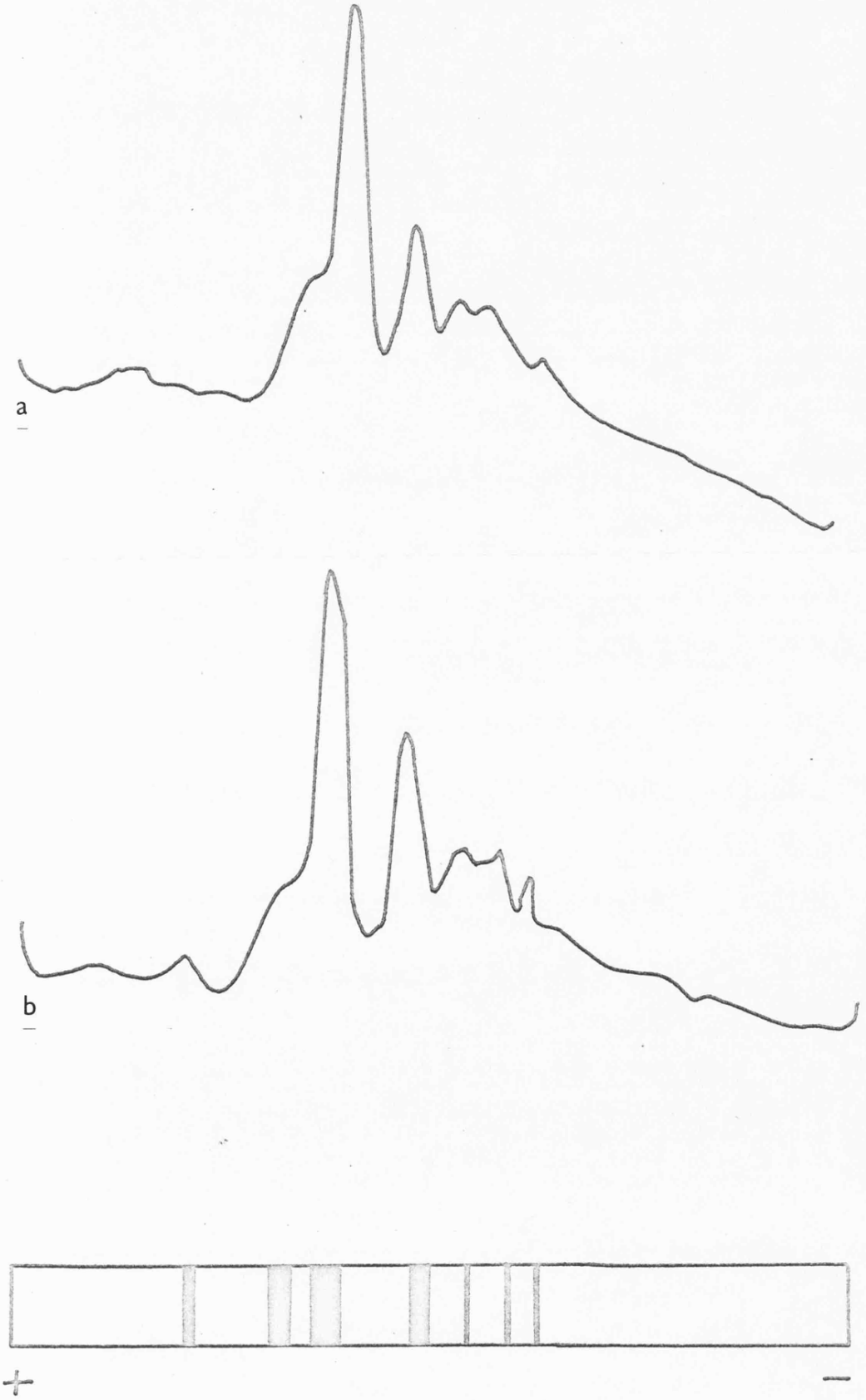

a
b
+
—

FIG. 37

Densitometer traces of Acid Phosphatase isoenzymes from light and dark grown cells of C.diospyri.

Conditions of scanning as described in Fig.33 except that the wedge used was coded 5-077C.

(a) light grown cells

(b) dark grown cells

Cells grown in Malt Broth at 25^{o} for 6 days.

a

b

FIG. 38.

Densitometer traces of Catalase isoenzymes from light and dark grown cells of C.diospyri.

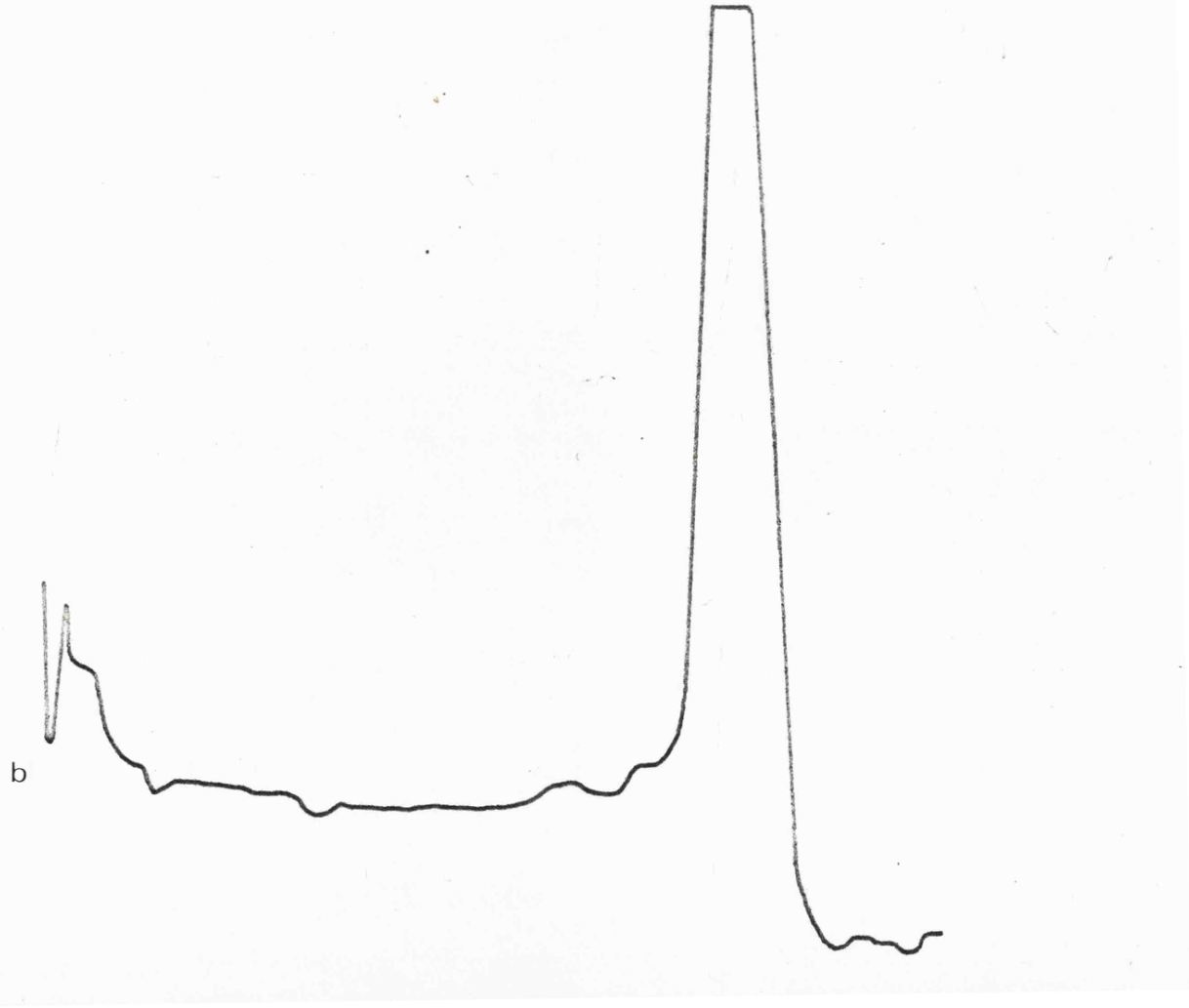
Conditions of scanning as described in Fig.34 except that the wedge used was coded 5-077C.

(a) light grown cells

(b) dark grown cells

Cells grown in Malt Broth at 25^{o} for 6 days.

a



b

grown cell extracts. Age of culture did not appear to have any qualitative effects on protein and esterase patterns. Cells grown in the light in Schopfer's medium for 6,8 and 10 days were analysed and their protein and isoenzyme patterns shown to be unaltered. A maximum of three catalase bands with Rf values of 0.29, 0.40 and 0.44 were detected on zymograms of all light grown and dark grown cell extracts (Fig.34).

In addition to this, cells of C.diospyri grown in the light and the dark on Malt broth and Czapek dox medium were also analysed for protein and isoenzyme patterns. Results in Figs. 35 and 39 show that protein patterns obtained, although different from each other and from those found in cells grown in Schopfer's medium, were identical in respective light and dark grown cells. Likewise, esterase patterns were unaffected by light (Figs. 36 & 40), although differing in cells grown in the three media.

In extracts from cells grown in these two media, the acid phosphatases although qualitatively the same, appeared to have pronouncedly altered relative activities in light and dark grown cell extracts (Fig. 37 & 41). Similarly, cells grown in the dark in Malt and Czapek Dox broth appeared to lack the fastest moving catalase band (Figs. 38 & 42). The reasons for these apparent differences are not known.

Peroxides in the growth medium.

Exposure of organic material to light causes in many cases non enzymic oxidation resulting in the formation of peroxides, and Weinhold & Hendrix (1963) have attributed the inhibition of growth of certain fungi by light to the presence of peroxides in illuminated medium. Therefore it was decided to analyse culture filtrates of cells of C.diospyri grown in the light and dark in Schopfer's medium for peroxides.

Peroxide concentration was measured by the method described by Hollomon (1966). 1ml of medium, 20ml of 95% (v/v) ethanol, 0.2ml conc. HCl and

FIG. 39.

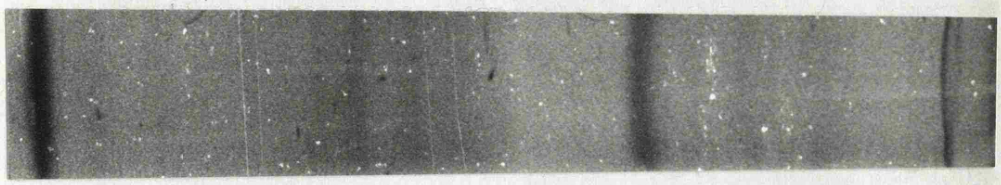
Densitometer traces of soluble proteins from light and dark grown cells of C.diospyri separated on polyacrylamide gels.

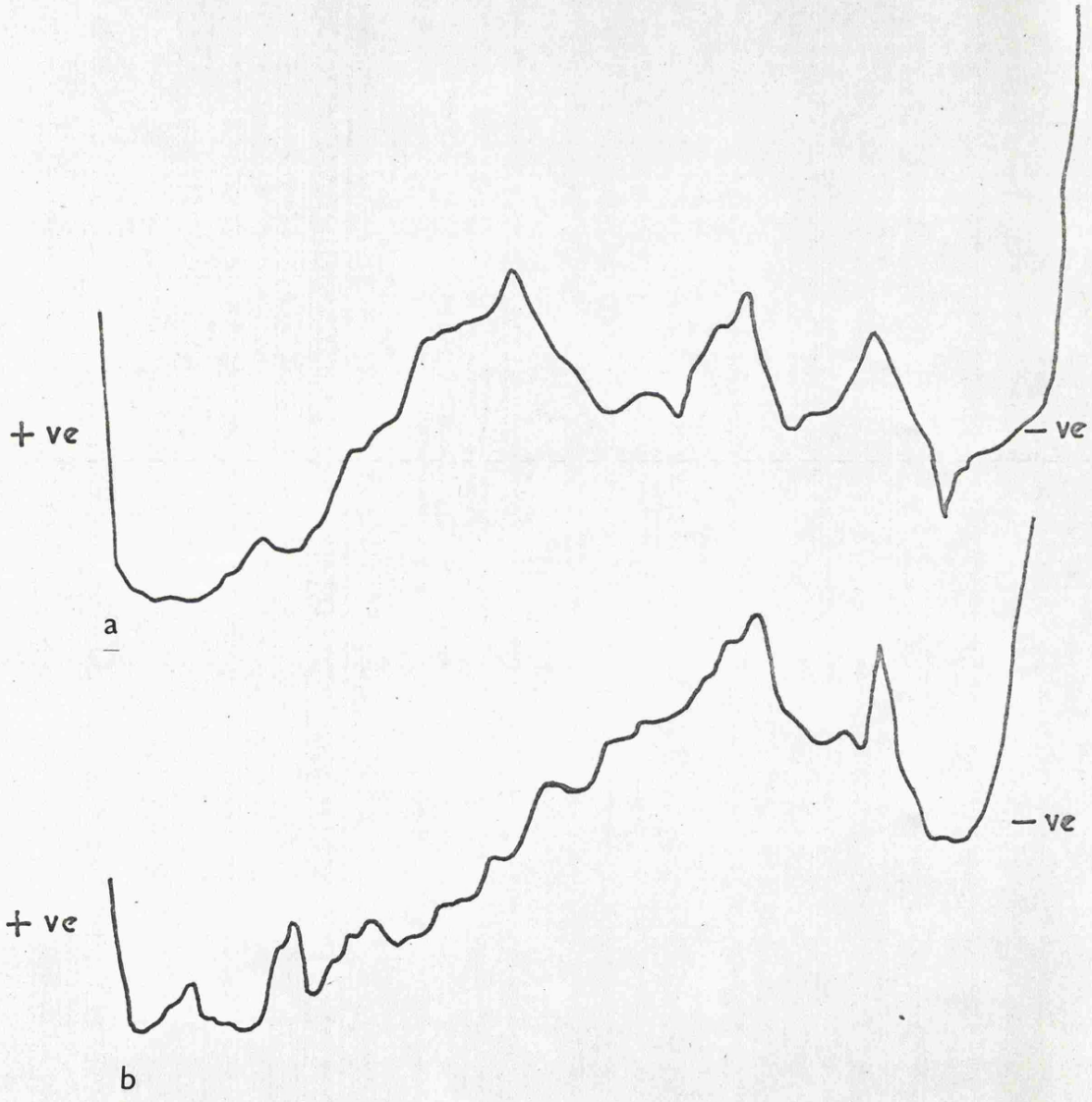
Conditions of scanning were as used in Fig.31.

(a) light grown cells

(b) dark grown cells

Cells grown in Czapek Dox Broth at 25^{o} for 6 days.




+ ve
– ve
a
– ve
+ ve
b

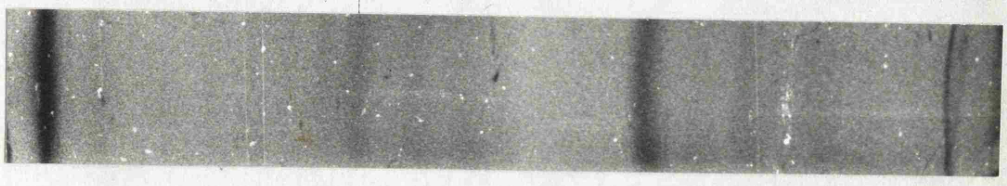


FIG. 40.

Densitometer traces of Esterase (acetate) isoenzymes from light and dark grown cells of C.diospyri.

Conditions of scanning described in Fig. 36.

(a) light grown cells

(b) dark grown cells

Cells grown in Czapek Dox Broth at 25^{o} for 6 days.

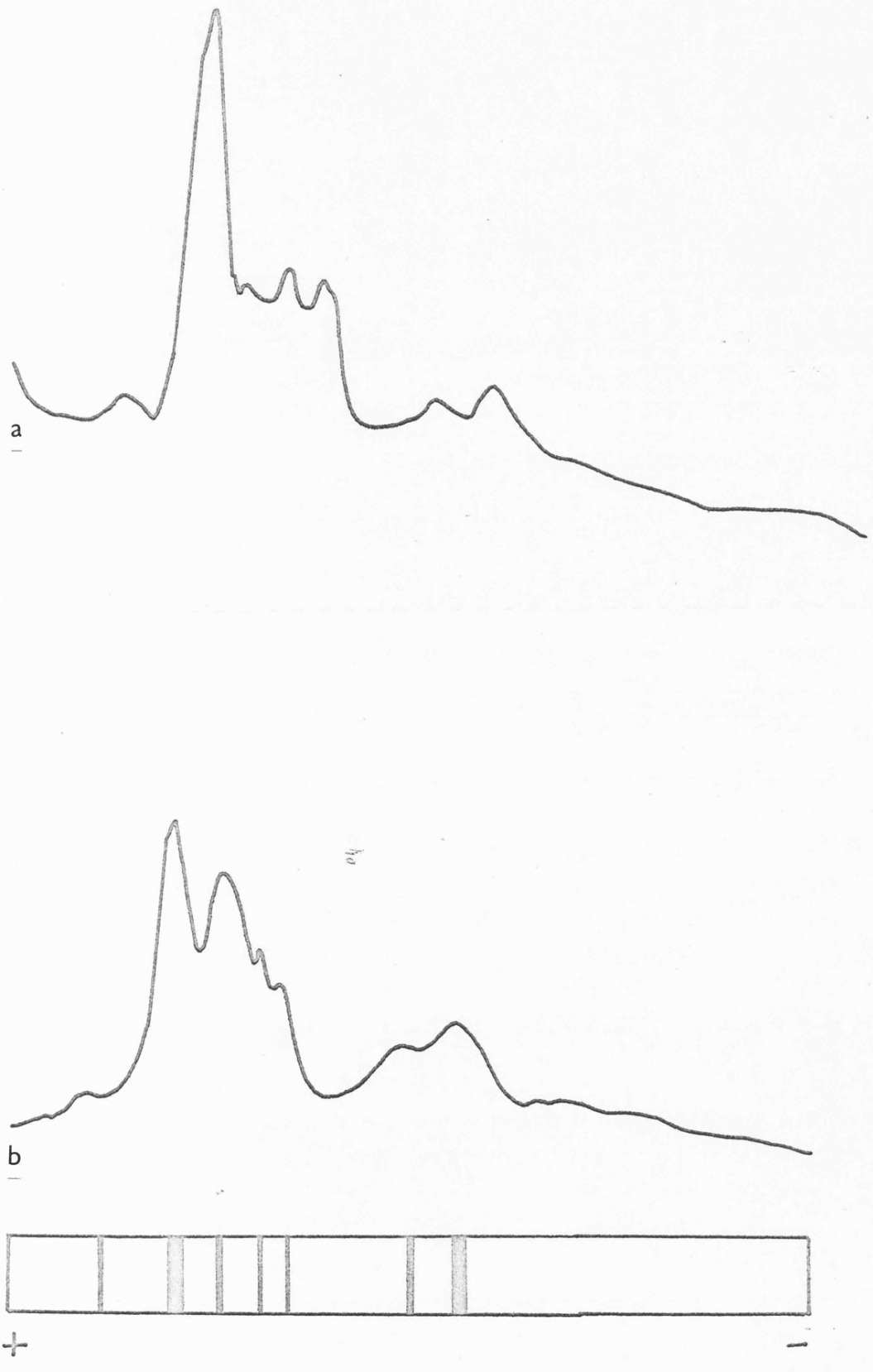

a
b
+
−

FIG. 41

Densitometer traces of acid phosphatase isoenzymes from light and dark grown cells of C.diospyri.

Conditions of scanning as described in Fig.37.

(a) light grown cells

(b) dark grown cells.

Cells grown in Czapek Dox Broth at 25^{o} for 6 days.

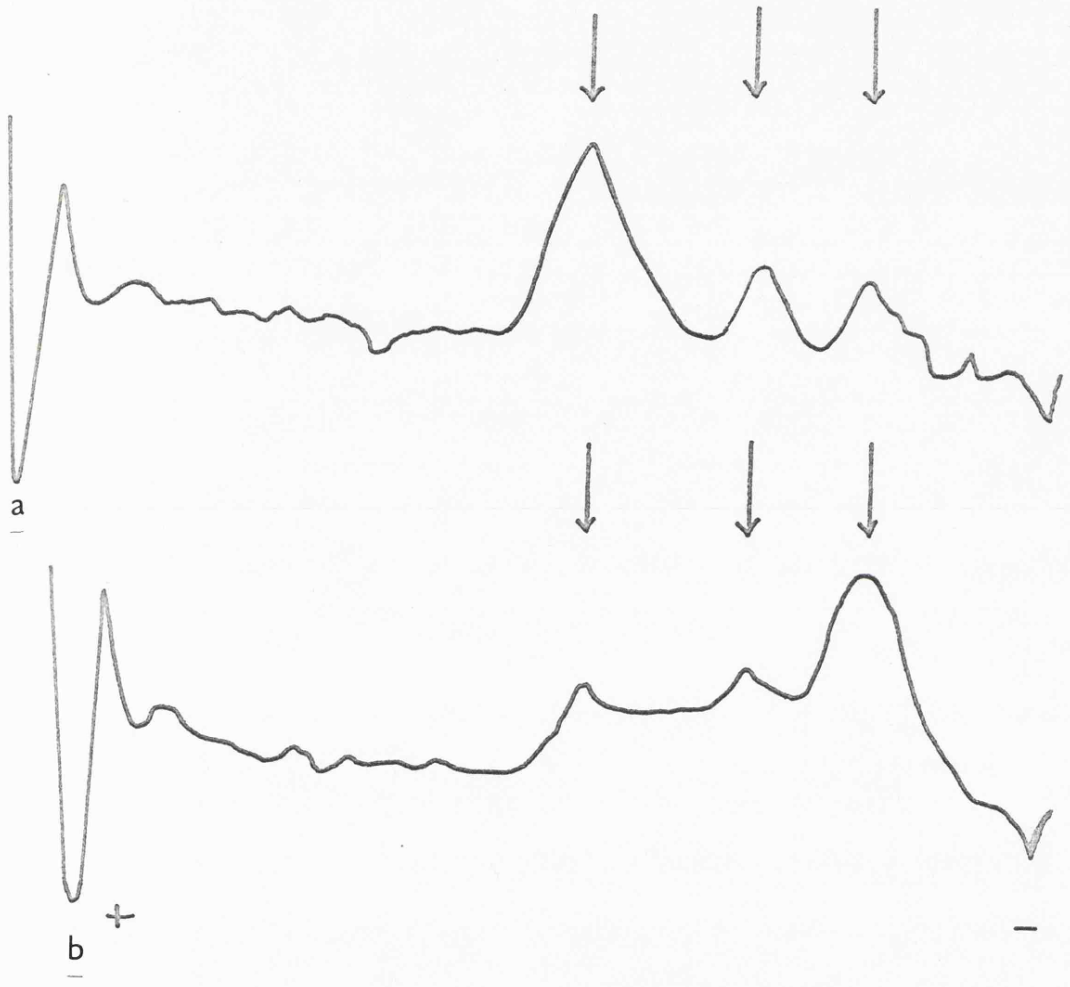

a
b
+
–

FIG. 42.

Densitometer traces of catalase isoenzymes from light and dark grown cells of C.diospyri.

Conditions of scanning as described in Fig.38

(a) light grown cells

(b) dark grown cells.

Cells grown in Czapek Dox broth at 25^{o} for 6 days.


a
b

0.05ml 5% (w/v) ammonium ferrons sulphate in 3% (v/v) HCl were shaken at room temperature for exactly 30 sec., and the alcohol insoluble glucan material removed with a spatula. 1ml of 20% (w/v) ammonium thiocyanate was then added, and after exactly 3 min. the absorbance of the red-brown solution measured at 480nm. This reading was compared with that obtained with freshly autoclaved medium.

No peroxides were detected in inoculated Schopfer's medium from both light and dark grown cultures over the growth period of 12 days. Similar results were obtained with Czapek dox broth.

Catalase Activity of cells of C.diospyri.

Catalase activity of cell extracts from light and dark grown cultures is shown in Table XVII. Corrected enzymic activity was the same in extracts from both. Boiled cell extract and buffer blank did not show any activity as measured by a drop in thiosulphate titre. The catalase activity of culture filtrates of cells grown in Schopfer's medium concentrated 5x by freeze drying were also determined and found to be negligible over the growth period in light and darkness.

TABLE XVII

Catalase activity of cell free extracts from cells grown under conditions of light and darkness in Schopfer's medium at 25^{o} for 6 days.

	Catalase activity $k_o \times 10^3$	
	1st Expt.	2nd Expt
Light grown	1.05 ± 0.10	1.12 ± 0.11
Dark grown	1.10 ± 0.14	1.07 ± 0.11

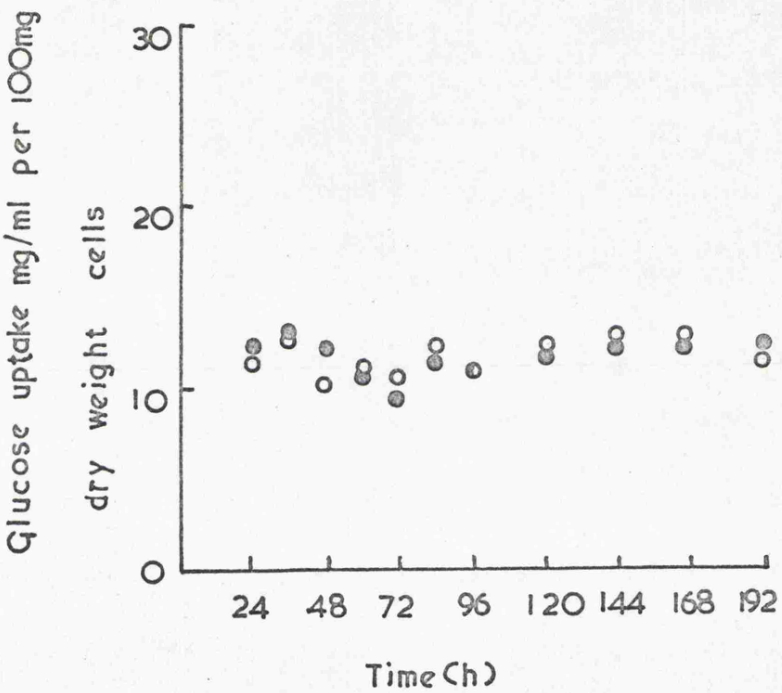
The velocity constant k_o was determined by extrapolation to t_o, and this value dixided by x/50 where x was the mg protein per test sample, to give the corrected catalase activity of the samples.

FIG. 43.

Consumption of glucose by C.diospyri. Cells were grown at 25° in a modified Schopfer's medium containing 2% glucose and 0.1% glycine. After harvesting at the stated periods of time, culture filtrates were analysed for glucose. Results are expressed as mgm/ml glucose consumed per 100mgm dry weight of cells.

o—o : light exposed cultures

●—● : dark cultures


Glucose uptake mg/ml per 100mg
dry weight cells
0
10
20
30
24
48
72
96
120
144
168
192
Time (h)

Effect of light on glucose uptake in C.diospyri.

Light has been reported to stimulate glucose uptake in Karlingea roseus (Haskins and Weston, 1950) and inhibit it in B.brittanica (Horenstein & Cantino, 1964).

Results in Fig.43 show that under the experimental conditions used, there was little or no difference in glucose uptake between light and dark grown cultures. In all the experiments performed, there was also no growth of the organism as measured by increase in biomass in the first 12h of incubation, due probably to the fact that the inoculum used was pregrown in Schopfer's medium. No emphasis is placed on these results since the experimental approach was felt to be unsatisfactory, measuring glucose uptake over such a long period with cells of such variation in physiological activity obtained with batch culture.

DISCUSSION.

The aim of this project was to investigate, in addition to the very obvious colour difference, possible alterations of metabolism brought about by exposure of cells of C.diospyri to light. The physiology of the form genus Cephalosporium has been very infrequently studied apart from the aspect of antibiotic production and this neglect has been stressed by Pisano (1963) in his review article. Therefore, as well as any attempt to study differences between light and dark grown cells, it was also necessary to study the metabolism per se. An effort has been made to record observations on various aspects of the metabolism of this fungus which were thought worthy of particular attention in the context of this investigation and in the light of previous reports concerning other fungi. It is hoped that these remarks may explain the very diverse approach taken in this thesis.

The biosynthesis of carotenoids in C.diospyri had an absolute requirement for light. Cells of C.diospyri grown under continuous illumination were found to contain phytoene, ζ-carotene, β-carotene, lycopene and torulene in the epiphasic fraction (Plate 3). Examination of dark grown cells, on the other hand, revealed only phytoene in any detectable amount. Rilling (1963) found that dark grown cells of an unidentified Mycobacterium sp contained both phytoene and phytofluene as well as an unidentified epiphasic pigment, which might suggest either different points of action for light in the biosynthetic pathway in these two organisms, or different pathways of carotenoid biosynthesis. The most probable explanation is the former. Phytoene is thought to be the first C-40 precursor formed in the biosynthetic pathway and not lycopersene, the compound theoretically formed from the dimerisation of geranylgeranyl pyrophosphate, the C-20 compound. Evidence has been obtained mainly from inhibitor studies that lycopersene was absent in the biological material investigated. For example, Rilling (1962)

could not detect lycopersene under anaerobic conditions in Mycobacterium sp., and suggested that its formation prior to phytoene which accumulated under such conditions would involve an oxidising step, whereas direct conversion of geranylgeranyl pyrophosphate to phytoene could theoretically occur with no overall change in the oxidation state of the system.

The results obtained with C.diospyri would appear to be in closer agreement with the situation reported for N.crassa. Zalokar (1954), however, did suggest that phytoene was formed by an independent pathway not related to any of the other C-40 compounds. Similarly, Karunkarun, Karunkarun & Quackenbush (1966) using N.crassa grown in continuous culture found with incorporation of C^{14} mevalonate that no pigment was synthesised under conditions of growth of the organism, and that the phytoene which formed and accumulated was an end product and not a precursor of carotenoids. Harding, Huang & Mitchell (1969) in a similar investigation have however questioned this theory on the basis of the inability of the previous workers to obtain pure carotenoid fractions and have concluded from their experimental data that phytoene does in fact act as a common precursor for the more unsaturated carotenoid pigments. The biosynthetic scheme suggested by Porter & Anderson (1962) postulates phytoene as being the main precursor, and the more unsaturated coloured carotenoids being formed, by a series of sequential dehydrogenations, from it. If this were the case in C.diospyri, then light would appear to allow the expression of an hypothetical enzyme, phytoene dehydrogenase, absent in dark grown cells. It could then be postulated that the products of this new enzyme, by a process of substrate induction, could then induce the enzymes necessary for their further conversion, and so on until all the enzymes necessary for the formation of all the carotenoids

were present. It is also possible that only one non-specific dehydrogenase or a single sub-divisible isoenzyme could be responsible for this series of reactions. Failure to detect phytofluene in light grown cells could be accounted for by its very rapid turnover rate in the biosynthetic sequence after its initial formation from phytoene.

The results obtained with C.diospyri for the light dependent production of carotenoids would suggest a process similar to those found in N.crassa (Zalokar, 1954), Mycobacterium sp. and Mycobacterium marinum (Rilling, 1964), and F.aquaeductuum (Rau, 1967), except that the organism used in this investigation appeared to be less sensitive to light, and the process proceeded at a much slower rate than in those organisms already studied. The overall production of carotenoids could, as in the previous reported cases, be sub-divided into three easily distinguishable separate stages, the first of which was the photoinduction which, unlike the subsequent steps, required light amounting to at least 30 min exposure to 2000 ft. candles (Fig.7). This primary stage was also temperature independent suggesting a photochemical reaction, and no induction of pigments was achieved under anaerobic conditions of light exposure (Table 6 & 7). As with N.crassa (Zalokar, 1954) and Mycobacterium sp. (Rilling, 1964), cells of C.diospyri possessed a memory for this light induction which could be manifested at least eight weeks after storage of the fungus at -20^{o} in the dark.

Recently, both Rau (1968) Trinci and Banbury (1969), working with different fungi, have reported that oxygen was not essential for the initial photoinduction stage, and Rau speculated that oxygen did not directly participate in the primary photochemical reaction, but acted as an electron acceptor maintaining the photoreceptor compound in the

proper oxidation state. In contrast however, Howes, Batra & Blakeley (1969) found that their two species of Mycobacterium were not photoinduced unless O_2 was present during the illumination, and the possibility exists that the conditions used by the other workers were not strictly anaerobic. Certainly C.diospyri appeared to have a critical requirement for oxygen both for the initial photochemical stage and the subsequent light independent stages, and it may further be suggested that this oxygen was directly participating in the photochemical reaction.

Both 'dark' stages after the initial one were temperature dependent and required oxygen for pigment production. Other workers (Rilling, 1964 , Rau 1967, Harding & Mitchell, 1969), have, by using inhibitors of protein synthesis, suggested the lag period encountered before actual carotenoid production in their organisms was a period of de novo protein synthesis. In the present work, chloramphenicol and cyclohexamide were tested for their ability to inhibit carotenoid production in C.diospyri and the latter compound was found to be effective (Fig.12), albeit in concentrations far higher (approx. 50x) than those which inhibited pigmentation in N.crassa and F.aquaeductuum (Rau, Lindemann & Rau-Hund, 1968). The exact stage in the overall process which was sensitive to cyclohexamide has not been elucidated. Other studies (eg. Rilling, 1964, Rau, 1968 and Harding & Mitchell, 1969) have suggested that light and oxygen acted by either photooxidising a repressor substance or by producing a photooxidised substance which then allowed the formation of enzymes responsible for carotenoid production. It would appear however, that the alteration of the metabolism did not result in the formation of products which could be readily transported from illuminated to non-illuminated parts of the fungus. It may also be pertinent that in the case of

the photomimetic compounds PHMB and PCMB, only fungal tissue directly in contact with them was induced to form pigment.

This hypothesis that carotenogenic enzymes were repressed in dark grown cells receives added support from the fact that carotenoid levels in cells of C.diospyri grown under conditions of continuous illumination contained approx. twice that amount found in cells initially photoinduced and then subsequently incubated in the dark (Figs 7 & 8). From this it might be surmised that the synthesis of carotenogenic enzymes was in a state of repression in dark grown cells, derepressed when exposed to light only to become gradually repressed again when reincubated in the dark. However, it must be remembered that any fresh growth of cells in the dark would result in non pigmented cells with a decrease in colour when the carotenoid content was expressed as production on a per 100 mg dry weight of cells basis. Conditions of incubation were chosen in this investigation which would avoid as much as possible this qualification.

Apart from pigmentation, light grown cultures of C.diospyri were easily distinguishable from those grown in the dark simply by the colour of their respective culture filtrates; those from light grown cells were pale yellow, fluorescing blue, as compared to filtrates from dark grown cultures which were strikingly green with very pronounced yellowish fluorescence. This was caused by the extracellular production of free riboflavin with the corresponding formation of a compound, identical in all properties studied, with those of lumichrome in filtrates from light grown cells (Figs. 18 & 19).

The physiology of riboflavin production by Eremothecium ashbyii in submerged culture has been studied by Kaprálek (1962). Evidence was

obtained which indicated a shift from a cytochrome type of terminal respiration to a flavoprotein type which was coupled to catalase. This induced flavoprotein was proposed to be an aldehyde oxidase, responsible for oxidising pyruvate, which accumulated in the culture medium because of incomplete glucose oxidation, to acetate via acetaldehyde, and it was this which was the alternative electron transport used after the loss of cytochromes at the commencement of riboflavin synthesis. There was a breakdown of the regulation of the flavin synthesis which caused over production of riboflavin. Changes in environmental conditions have been known to alter mechanisms of electron transport, since Lenhoff, Nicholas & Kaplan (1956) found with Pseudomonas fluorescens that iron deficiency caused an increase in the activity of flavin enzymes with a decrease in activity of the cytochrome system. By comparisons of the mechanism of riboflavin synthesis in E.ashbyii and Ashbya gossypii (Mickelson 1950), Kaprálek suggested that the organisms had very similar methods of biosynthesis, and pyruvate played the role in E.ashbyii than ethanol did in A.gossypii.

From preliminary results obtained with C.diospyri it would seem that the process was very much like that described for E.ashbyii. In both organisms, pyruvate was found to accumulate in the growth medium (Fig.29) although in the case of C.diospyri, α oxoglutarate was also detectable, which was not the finding of Kaprálek (1962) with E.ashbyii. These keto acids disappeared at the onset of riboflavin production in the medium. No attempt was made to follow catalase activity over the period of riboflavin, but examination of both light and dark grown cells of C.diospyri actively synthesising riboflavin by direct vision spectroscopy failed to reveal any characteristic absorption bands due to cytochromes, even when examined

at the temperature of liquid nitrogen and reduced with sodium dithionite (Boulter & Derbyshire, 1957). If verified by more detailed study, this would be further evidence for an alternative electron transport pathway operating during riboflavin synthesis in C.diospyri.

There has been no indication that light and dark grown cells of C.diospyri differed in their ability to synthesise riboflavin. On the contrary, cells grown in the light when replaced in darkness immediately began to accumulate riboflavin in the culture medium (Fig.21). Also, the factors implicated by Kapralek to be involved in riboflavin production were, where measured, shown to be the same in both light and dark grown cultures. C.diospyri, unlike E.ashbyii did appear to possess a considerable intracellular pool of free riboflavin (see later) and neither FAD nor FMN were detected in aqueous extracts of cells. It is believed that this is the first recorded instance of a fungus which produced carotenoids in light and also produced an excess of riboflavin in the dark. The possible connection between these two observations should be considered, especially since riboflavin has been suggested to be a trigger photoreceptor substance for the initiation of carotenoid synthesis. In addition, one of the prime objections raised by Ingold (1962) against a flavoprotein receptor was that riboflavin had not been detected in any photosensitive fungus. In this investigation unfortunately, it has not been possible to separate riboflavin synthesis from carotenogenesis. However it is known that lumichrome, present in culture filtrates of light grown cells is a competitive inhibitor of flavoprotein synthesis. Carlile (1962) has in fact suggested that there were different electron transport mechanisms in light and dark grown static cultures of P.blakesleeanus, the former including as a component a flavoprotein

susceptible to inhibition by lumichrome. On this basis then, it is quite possible that lumichrome might be directly involved in pigmentation in C.diospyri whereas free riboflavin which is photolytically active, was the photoreceptor in dark grown cells involved in the suppression of that electron transport pathway. It is of interest that Thiemer & Rau (1970) suggested such an alternative mechanism for carotenoid synthesis in dark grown cells which, on exposure to light, was inhibited in F.aquaeductuum, although any evidence for such an alternative mechanism of pigmentation has not been obtained for C.diospyri. Certainly one might expect lumichrome to have an effect on the metabolism of the fungus, if permeable to it, but attempts to substitute this compound for light in carotenogenesis in submerged culture were unsuccessful. However, the experience obtained in applying other photomimetic compounds to C.diospyri could possibly indicate that these growth conditions were not suitable for lumichrome to be effective. Although, as mentioned already, no lumichrome was detected in extracts from light grown cells of C.diospyri.

The photoreceptor for the production of carotenoids has thus not been identified. On the basis of the premise that its presence would be detectable in dark grown cultures, the evidence, again, like that presented by many investigators is confusing and in the main negative rather than constructive. Carotenoids were not present in any detectable amounts in dark grown cells, and it would be difficult to envisage how they could be involved in their own photo-induction. Methanolic and aqueous extracts from dark grown cells were found to contain a yellowish pigment with an absorption spectrum (Fig.23) similar to that found in dark grown cultures of Aspergillus giganteus (Trinci & Banbury, 1969), and proposed by them to resemble chrysogenin.

An investigation of the chemical properties of this compound by Wolf et al (1960) has on the evidence of its infra red spectrum indicated it to be 2-methyl-4,5-dihydroxy anthraquinone which fluoresced under ultra violet light with a broad emittance maximum of 460-540nm. Fluorometric examination of the extracts from dark grown cells of C.diospyri however, showed a sharp emittance maximum of 530nm (Fig.23), chromatographic properties identical to riboflavin and similar to aqueous extracts from light grown cells. Zalokar (1955) found that ice-cold water extracts from dark grown cells of N.crassa also gave a yellow solution which, on purification had an absorption maximum of 410nm. There was no mention of its fluorescent properties if any, though it is worth remembering that complexing either FMN or FAD with a protein as in most flavoproteins results in a loss of fluorescence (Oster, Bellin & Holmstrom, 1962). For this reason, as well possibly, the absorption spectrum of this compound did not correspond to the action spectrum of the photoresponse. Flavins were suggested as photo-receptors in this system, although the total levels of riboflavin in illuminated and non-illuminated mycelium were identical, results in agreement with those obtained for C.diospyri in this investigation. Flavins have also been implicated as photoreceptors in Mycobacterium sp by Howes & Batra (1969) who found in extracts from dark grown bacteria a yellow flavin like compound which fluoresced and possessed an absorption spectrum with maxima at 290, 357 and 445nm, corresponding to the action spectrum for carotenogenesis in the Mycobacterium.

Only light of a wavelength less than 470nm was capable of inducing carotenoid formation in C.diospyri (Table 8). More detailed action spectra obtained from carotenogenesis in N.crassa (1955), F.aquaeductuum (Rau, 1967) and Mycobacterium sp. (Howes & Batra, 1970) have indicated the participation of flavins or flavoproteins as the

photoreceptor molecules. This hypothesis was based mainly on the appearance of an absorption maximum in the action spectrum in the region about 370nm. In a recent publication however, Hager (1970) has reported that the addition of definite amounts of water to carotenoids in polar solution changed the absorption spectrum of them in a characteristic fashion. A new peak appeared in the near ultraviolet region of the spectrum, in some cases around 370nm, caused, he suggested, by an aggregation of the carotenoid molecules with a concomittant change in the absorption spectrum. Hager further proposed that solutions of carotenoids which did not show a peak at 370nm would do so if bound to membranes and subcellular particles. That this might be so was inferred in a report by Hager & Pertz (1970) who isolated an enzyme substrate complex responsible for the dehydrogenation of violaxanthin. They found that under these conditions the absorption of violaxanthin was drastically changed, the normal three peak absorption curve in the blue region of the spectrum was strongly decreased, with the appearance of a new maxima around 380nm. It was suggested that part of the carotenoids were present in plant tissue in protein bound form and these pigments showed considerable difference in light absorption in comparison to the isolated carotenoids. Certainly several other workers eg. Thirkell & Strang (1967) have also presented evidence to suggest that carotenoids could be present as complexes with proteins or polysaccharides. Thirkell *et al* (1969) have isolated a carotenoid glycoprotein from *Sarcina flava*, and Thirkell & Hunter (1969) have in fact proposed a model to show the incorporation of carotenoids in the membrane. No information has been obtained as to the regional location of carotenoid pigments in *C.diospyri* but carotenoids in *D.spathularia* were found to be concentrated at the cell wall or the plasma membrane (Vail & Lilly, 1968).

The similarity of many action spectra to certain of the 4-peak carotenoid spectra described by Hager (1970) is remarkable, including those for light induced carotenogenesis in organisms where flavins have been proposed. This, together with the non-correlation of action spectra with the absorption spectrum of the proposed flavin explained in terms of differences between the optical properties of the compound in vivo and in vitro, might encourage a reappraisal of photoreceptor molecules and action spectra. Recently however, crystals of riboflavin arranged in an orderly fashion have been detected in the sporangiophore of Phycomyces just below the light sensitive region (Wolken, 1969). Mepacrine, a flavoprotein inhibitor of a very doubtful specificity has been found to have no inhibitory effect on carotenogenesis by C.diospyri. and, it would appear, little effect on riboflavin production. There was also no difference in inhibitory effect on the growth of C.diospyri in Schopfer's medium in either light or darkness (Fig.28). These results like those of Hocking's (1963) would add further doubt to the claim of Carlile (1962) based on observations obtained with P.blakesleeanus suggesting a flavoprotein photoreceptor, and it would indicate that the results he obtained were in part, due to nutritional conditions. Many investigators have used mepacrine (eg. Tschabold, 1967) as indicative of flavin involvement in the photoresponse, but because of this lack of specificity a possible flavin photoreceptor in C.diospyri should not be ruled out because of the negative results obtained with mepacrine in this study.

Apart from carotenoids which would seem unlikely, the other class of compounds which have been suggested are porphyrins. The microorganisms proposed to use them as photoreceptors, however, responded to light of a much longer wavelength, above 500nm (Howes & Batra, 1969; Burchard & Hendricks, 1968). Certainly this criterion would eliminate the possibility of them acting in this way in C.diospyri unless they too in

complex with other compounds change their optical properties sufficiently to absorb light in the appropriate region of the spectrum *in vivo*.

Carotenoid synthesis is known to be inhibited by a wide range of compounds in microorganisms (Rilling, 1965), but many eg. cyclohexamide, are inhibitors of other metabolic processes with carotenogenesis affected indirectly. Diphenylamine, the most commonly used, has caused much speculation as to its precise mode of action. For example, Turian (1957) suggested that the inhibitory action was indirect, since it was known to possess antioxidant properties. On the other hand, Olson & Knizley (1961) have found that under conditions of carotenoid inhibition in *P.blakesleeanus*, diphenylamine did not affect either sterol synthesis or fatty acid synthesis, and because of this concluded that diphenylamine was specific in inhibiting carotenogenesis. Only microorganisms which synthesise open chain carotenoids or synthesise no hydroxylated carotenoids are affected by diphenylamine, and Rilling (1965) has proposed that as diphenylamine had the same molecular structure as the central portion of phytofluene, it might inhibit carotenogenesis by binding with the dehydrogenase responsible for the initial unsaturation. Further confusion however arises from the knowledge that diphenylamine was itself capable of photooxidation to carbazole (Grellman *et al*, 1963), with the production of a stable intermediate. The carbazole produced is structurally similar to acridine shown also by Rilling (1965) to inhibit carotenogenesis in a *Mycobacterium sp*. and thought to prevent the production of M-RNA. Thus diphenylamine could well be acting indirectly earlier in the biosynthetic mechanism than the site suggested by Rilling (1965). Although no detailed analysis of pigments was performed, the absorption spectra from extracts of inhibited cells (Fig.11) of *C.diospyri* did

suggest the gradual disappearance of the less saturated carotenoids, and, further evidence that only after the initial photooxidation stage was diphenylamine effective in inhibiting carotenogenesis would support the view that it was the later stages of carotenogenesis which were sensitive. This observation, in agreement with that reported by Zalokar (1957) for N.crassa, would rule out the possibility that diphenylamine was acting because of its antioxidant properties.

However, the effect of diphenylamine was not restricted in C.diospyri to carotenogenesis. Riboflavin synthesis was also found to be inhibited in dark grown cultures in the same range of concentrations of diphenylamine which inhibited pigmentation in light grown cells. (Fig.24). Failure to detect riboflavin was not due to any chemical alteration of it by diphenylamine since none of the normal degradation products like lumichrome or lumiflavin were revealed by analysis, and a riboflavin solution when incubated with diphenylamine in the dark did not lose any of its fluorescence. This is, as far as can be gauged, the first recorded instance of riboflavin production inhibited by diphenylamine, and as well as casting further doubt on the exact mode of action of the inhibitor, could possibly help explain the results obtained by Goodwin et al (1953) who partially reversed the effect of diphenylamine by adding riboflavin to diphenylamine inhibited cells of P.blakesleeanus. As far as pigmentation is concerned it should also be noted that in light grown cultures of C.diospyri, the presence of diphenylamine could by preventing the formation of lumichrome which may be involved in carotenogenesis, indirectly affect the pigment formation.

The mechanism of inhibition of riboflavin synthesis by diphenylamine has not been elucidated, but it is quite possible that catalase is directly involved. This enzyme, considered by Kapŕalek to play an

important role in flavin synthesis in E.ashbyii, and which was inducible in Rhodopseudomonas spheroides, was found (Shanmugam & Berger, 1969) to be non-induced in cells of R.spheroides incubated in the presence of diphenylamine. Therefore, if catalase formation could be shown to be inhibited by diphenylamine, by analogy with the results of Kapŕalek (1962), no riboflavin would be synthesised in C.diospyri. Mitchell & Anderson (1965) have reported that catalase activity was identical in pigmented cells of Sarcina lutea grown in the light and dark, results in agreement with those obtained in this work with C.diospyri, grown in Schopfer's medium. However in carotenoid deficient cells of S.lutea and corn (Zea mais), they found that under aerobic conditions, catalase was photoinactivated by an alteration of one porphyrin moiety in the catalase molecule. Furthermore, visible light (405nm max.) was responsible, and their proposal was that carotenoids prevented the inactivation of this porphyrin which photosensitised its own destruction. The destruction of other porphyrins by light was reported by Ninnemann et al (1970) who found that irradiation of Saccharomyces cerevisiae which did not possess any carotenoids, resulted in the destruction of cytochromes a. and a_3, and partial destruction of cytochrome b. Therefore, it would appear that carotenoids do possess a large potential as protective agents against light damage as suggested by workers previously, and it would be of interest to investigate porphyrin content of light and dark grown cells of C.diospyri incubated with an inhibitor of carotenogenesis such as diphenylamine.

Suggestions of possible mechanisms by which carotenoids could serve as protective agents would include their ability to quench free radical formation (Fujimori & Tavla, 1966) and this might explain why Thiemer & Rau (1970) were able to induce carotenogenesis in dark grown cells of F.aquaeductuum incubated with hydrogen peroxide. This compound,

when tested in C.diospyri was not capable of substituting for light, although peroxides were thought to be involved in the light response in C.diospyri since it is also known that light is capable of producing singlet O_2 by photosensitisation of redox compounds especially flavins and cytochromes (Foote & Denny, 1968). Theimer & Rau (1970) suggested then, that hydrogen peroxide simulated the oxidative activity of light since they also reported that dithionite and hydroxylamine inhibited light dependent carotenogenesis in F.aquaeductuum. These two compounds were not tested with C.diospyri, since like other inhibitors, dithionite at least is capable of acting in an indirect manner, and it is known that riboflavin and cytochromes are both very rapidly reduced by this compound. In fact, Batra & Rilling (1964) on the basis of the inhibition of the photoinductive reaction in carotenogenesis by dithionite proposed a flavin photoreceptor in Mycobacterium sp.

Possible changes in redox potential have been used to explain the effect of light on fungi. For example, Hollomon (1956) attributed the decrease in growth of Phytophthora infestans on illuminated medium to changes in its reduction potential, since both -SH group containing compounds as well as ascorbate overcame the inhibition due to light. With reference to carotenogenesis, the radiation energy would be inadequate by itself for pigment production in C.diospyri and a mechanism for amplifying and translating the light signal to the mechanism for their synthesis could well involve metabolic changes in redox potential as suggested by Klein & Edsall (1962) for phytochrome mediated photomorphogenesis. Such a metabolic series of changes might well operate in C.diospyri, since the presence of extracellular riboflavin would on illumination produce a significant initial change in the redox potential of the medium and also production of free radicals, although none could be detected in the system used in this investigation.

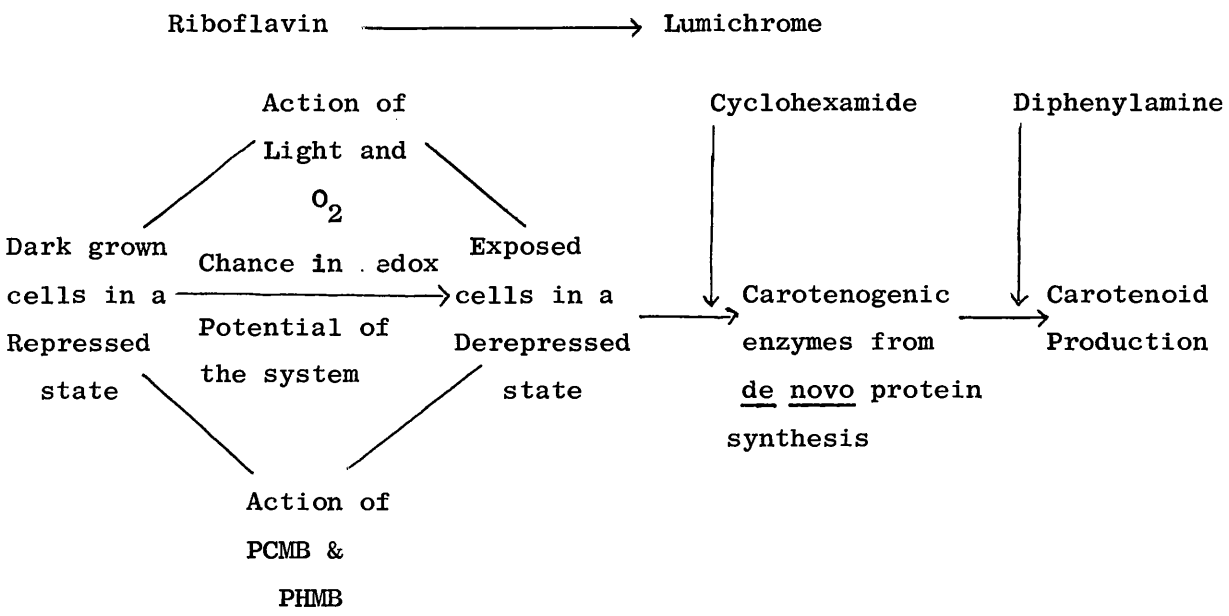
Antimycin A, which substituted for light in Mycobacterium marinum, an organism whose action spectrum was characteristic of a porphyrin (Batra, 1967; Howes & Batra, 1970), was not photomimetic in C.diospyri, and in fact at higher concentrations inhibited both growth and carotenogenesis. As this compound is an inhibitor of cytochrome mediated respiration (Batra, 1967), it might be presumed that cytochromes were present and involved in carotenogenesis in C.diospyri. However the specificity of Antimycin A. has been queried, and cells of Bacillus megaterium treated with this antibiotic were found to have impaired function of the plasma membrane (Marquis, 1965). In addition, Batra (1967) thought it unlikely that Antimycin A initiated an alternate electron transport mechanism for induction of pigments, since another inhibitor 2N-heptyl-4-hydroxyquinoline-N-oxide which blocks electron flow at the same site as Antimycin A had no effect on carotenogenesis in M.marinum. The situation in C.diospyri would appear to be more similar to that reported for F.aquaeductuum (Rau, 1967). However, from a selection of -SH group inhibitors tried, only PCMB and PHMB substituted for light in carotenogenesis in C.diospyri, and then only when the organism was grown on solid medium. As in light dependent carotenogenesis, only those portions of the fungal tissue directly in contact with the stimulus produced pigments. Growth of C.diospyri as measured by increase in colony diameter was inhibited by these compounds, more so by PHMB than PCMB, and the concentrations needed for the response were higher than in F.aquaeductuum. ie. at least 5×10^{-5}M as compared to 5×10^{-6}PHMB, when incorporated into solid medium. No fungus other than F.aquaeductuum has been reported which responded to these compounds in this manner, unusual when one considers the number of microorganisms which respond to light by producing carotenoids, but from experience obtained in this study, it

could well be that if tried, the cultural conditions were not suitable for the test organisms to respond. The role of -SH groups in the metabolism of an organism is an important one, not the least of which is their potential for photooxidation (Woodward, 1933), and theoretically there are many possible sites of action for these photomimetic compounds. In fact Rau (1967) tentatively proposed a number of -SH containing flavoproteins which also possessed action spectra very similar to that of the photoresponse in F.aquaeductuum, notable amongst which was lipoyl dehydrogenase. Since it is now thought that the sites of action of light and the mercuribenzoates were different, this suggestion loses some of its significance. It is also known that certain enzymes in the biosynthetic chain leading to production of carotenoids contain -SH groups, for example the enzyme responsible for the conversion of geranyl pyrophosphate to phytoene (Tung-Ching Lee & Chichester, 1969). How their inhibition would result in the production of carotenoids is not known. When one considers the redox potential of the cell, there is the chance that alteration of the sulphydryl-disulphide balance by these compounds could, like the photooxidation of -SH groups, as suggested by Klein & Edsall (1962), possibly cause pigmentation by amplification of a 'dark' carotenoid pathway suggested to occur in cells of F.aquaeductuum. Evidence obtained would however suggest that this is not the case in C.diospyri.

Because of the inability to obtain pigmentation in liquid cultures of C.diospyri with these compounds, the experimental work attempted with C.diospyri was unavoidably limited. Certainly there remains an enormous scope for investigation in this area, where these problems and many others in this field would be solved by the development of a workable cell free system. Substantial practical

difficulties remain to be surmounted before such a system would be of meaningful application, not the least of which would be the insolubility of the C-40 carotenoids in aqueous solution. However, the use of crocin, a water soluble carotenoid glycoside, used by Friend & Acton (1966) as an artificial substrate for dehydrogenation reactions could be envisaged.

Based on these results and other work, a schematic representation of the light dependent carotenogenesis in C.diospyri may be constructed as shown below:



Because light was responsible for carotenoid production in C.diospyri and because it was considered that as such a small portion of the overall metabolism of the organism would be involved in their synthesis (carotenoids contributed approx. 0.03% w/w to the mass of C.diospyri), the intriguing possibility remained that other more major aspects of the metabolism of C.diospyri could also be considerably affected. This supposition was further supported by the fact that light is known to modify chemically a large number of organic compounds with in some cases, formation of free radicals (Pryor, 1970).

Pyrolysis of samples of light and dark grown cells of C.diospyri gave patterns which were qualitatively identical, but with quite noticeable differences in their relative peak heights. The distribution of pyrolysis products has been shown to be directly related to the chemical composition of microorganisms (Reiner & Ewing, 1968), but which specific chemical structures or combinations contribute to the total pyrollysis profile is not yet known. The patterns obtained for C.diospyri appeared to be much more complex than those described by Meyers & Watson (1968) for their fungi. However the profiles obtained would suggest that the chemical composition of light and dark grown cells of C.diospyri differed. The sensitivity of this technique may be gauged from the claim by Reiner & Ewing (1968) to have differentiated between two strains of E.coli only differing in a single gene mutation. Attempts to determine gross differences in the metabolism of C.diospyri induced by light were largely unsuccessful, and it would appear that in many physiological aspects of metabolism, light appeared to be without effect. It was considered that cells analysed after exposure to continuous illumination (2000ft candles) for 6 days would illustrate any such changes.

The effect of light on the growth of C.diospyri, measured as total biomass was variable in submerged culture, even though care was taken to standardise inocula, but in most cases there was slightly less total dry weight from illuminated cultures. When growth was defined on slightly more rigid terms however, the residual dry weights expressed as percentages were the same in both cases over the growth period. Together with results obtained for chemical analysis where parameters measured also showed no difference, it might imply similarity in overall synthetic capabilities of light and dark grown cells of C.diospyri.

As mentioned earlier, both sterols and carotenoids are formed from isoprenoid units and share a common biosynthetic pathway up to and including the formation of farnesyl pyrophosphate. The cell free system for P.blakesleeanus used by Yokoyama et al (1962) allowed an interesting compartmentalisation of sterol and carotenoid synthesis to be hypothesised. They suggested that NAD was required for the formation of sterols but not carotene biosynthesis. If true, this might suggest that if there were differences in availability of NAD between light and dark grown cells of C.diospyri, their sterol content might differ. In fact, sterol content of the cells were the same (Table XIV.), results in agreement with those obtained with Epicoccum nigrum (Gribanowski-Sassu & Foppen, 1968).

Whether a situation similar to that which exists in B.emersonii (Cantino & Horenstein, 1956; 1959) occurs in C.diospyri, the evidence so far obtained would suggest not. Where investigated, the correlation between the light and dark metabolism in these two fungi was nil, as can best be seen from the following comparisons.

(i) Light grown cells of B.emersonii (BEM) contained less carotenoid pigment than dark grown cells. The opposite was true of C.diospyri.

(ii) There was no qualitative difference in the distribution of amino acids in light and dark grown cells of B.emersonii the same as found with C.diospyri. However, the amino acid pool of light grown cells of B.emersonii was much less than in dark grown cells (Cantino, 1959), due to the incorporation of soluble nitrogen into alcohol insoluble proteins and chitinous material in the light. No obvious quantitative difference was noted in the separated amino acid pools of C.diospyri between light and dark grown cultures, and T.C.A. soluble nitrogen was the same in both cases.

(iii) Light grown cultures of B.emersonii produced up to 300% more total biomass than dark grown cultures. No such

increase was noticed for submerged cultures of C.diospyri.

(4). Light grown cultures of B.emersonii fixed CO_2 via ∝oxoglutarate in an NADP mediated reductive carboxylation. No attempt was made to determine the extent of, and differences in CO_2 fixation in light and dark grown cultures of C.diospyri, but with this situation in B.emersonii, there was an increase in succinate with a concommitant decrease in ∝oxoglutarate in light grown cells as compared to dark grown cells. Certainly, from separated chromatograms of intracellular organic acids, this did not appear to be the case with C.diospyri, and analysis of cells showed ∝oxoglutarate was present in the same amount in both light and dark grown cells.

One of the major disadvantages suffered in this investigation was the fact that nothing is known of the mechanism of glucose metabolism by C.diospyri, and it is felt that before any meaningful conclusions can be drawn from a study of this type, this topic should receive primary attention. In addition one might be advised to employ short term incubations especially with labelled substrates, where the immediate effects of light exposure, if any, would be demonstrated with greater certainty. After 6 days growth of the fungus, sequential events due to such treatment would be very difficult to interpret.

Comparison of both the amino acid pools and the keto acid content of C.diospyri with those found for E.ashbyii (Osman & Chenouda, 1965), organisms which as mentioned previously both synthesise riboflavin, allows better interpretation of the results obtained. In both, the amino acid pools did not change qualitatively over an 8 day growth period, and in neither was any amino acids liberated in detectable amounts into the culture medium. Also, unlike Kapŕálek (1962) these workers reported the presence of ∝oxoglutarate in culture filtrates of

E.ashbyii, results in agreement with those found in this investigation with C.diospyri although in both instances ∝oxoglutarate was present in very much smaller amounts than pyruvate. However, unlike the situation reported for E.ashbyii, the amount of pyruvate per unit dry weight of cells of C.diospyri was less than ∝oxoglutarate (Table XV). These results are very unusual, especially when Sprecher (1961) found that with glucose as the carbon source, the fungi he analysed contained more pyruvate than ∝oxoglutarate. The reasons for this discrepancy are not known, but the assay system for keto acids differed in this investigation from that used by Osman & Chenouda for E.ashbyii.

Organic acids detected from both light and dark grown cells of C.diospyri, were most of those commonly associated with the tri-carboxylic acid cycle. Also present in quite appreciable amounts in both extracts and filtrates from cultures of this organism was an acid identified tentatively as itaconic acid, proposed to be synthesised by the decarboxylation of cis-aconitic acid in Aspergillus terreus (Bentley & Thiessen, 1957).

The amino acid sequence of protein molecules is a direct translation of the genetic information carried in the DNA which determined this sequence. Thus any technique which measured the physical properties of a protein resulting directly from the amino sequence such as the isoelectric point and molecular size should reflect genetic expression. As de novo protein synthesis has been proposed for light induced carotenogenesis in the microorganisms studied, it was decided to compare electrophoretically, separated soluble proteins from light and dark grown cells of C.diospyri, in the hope that such proteins might be detected. No visible qualitative difference was apparent between those cultures exposed to light and those maintained in darkness from the three media used. Subden & Turian (1970) by examining in the same

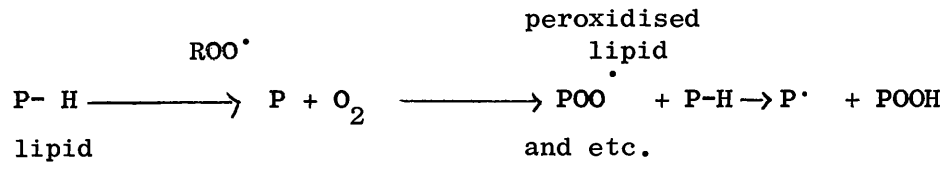
manner soluble protein composition of light induced and non-induced cultures of N.crassa have detected an extra protein band in the light exposed cells, which they suggested was the carotenogenic protein, although the same protein could not be detected immunologically. This apparent difference from C.diospyri could be partially due to the fact that N.crassa was much more sensitive to light than C.diospyri. However it might be considered unusual that such an enzyme would be in a soluble form intracellularly, and not membrane bound. Even so, the method employed to obtain extracts for electrophoresis from C.diospyri would have been sufficiently vigorous to allow physical solubilisation of any such enzyme to have occurred.

Only catalase isoenzymes from cells of C.diospyri grown on both Malt broth and C.dox broth differed due to light. The reason for the extra catalase band found in light grown cells is not known, but the possible significance of catalase in the metabolism of C.diospyri has already been considered. Why cells grown in Schopfer's medium did not illustrate this apparent difference is also unusual although the histochemical assay used for detecting catalase isoenzymes leaves much to be desired. The only other report which has considered the effect of light on protein and isoenzyme patterns in fungi was that of Hall (1966) using starch gel electrophoresis, and his findings with Fusarium solani agreed with those obtained for C.diospyri, in that light had no effect on the protein patterns obtained.

Critical assessment of this technique would suggest that there is enormous scope for artefact formation. These would include the variability in uptake of naphthalene black by proteins, meaning that stain intensity differences between bands may not be a reliable quantitative criterion. Also, protein-buffer interactions could result in single proteins separating as multiple bands and in the case

of dehydrogenases, positive results have been obtained with no added substrate (Shannon, 1968). Interpretation of both photographic and densitometer trace records of protein patterns has caused great difficulty, especially in taxonomic work, there the required degrees of similarity are a problem to estimate. Densitometer traces used in this investigation showed large quantitative differences even between two gels of the same protein extract, even though visibly they were identical. It is thought then that, with the exception of isoenzyme patterns, better comparative analysis could be made from photographic records.

Light has been implicated in the ageing processes of tissues especially animal, brought about by a number of mechanisms. This topic has been discussed in an article by Pryor (1970). As already mentioned, biological oxidations, in many instances, involve the formation of free radicals and it is known that flavin containing enzymes, on photooxidation, produce stable radicals involved in the formation of hydrogen peroxide. Possible mechanisms whereby light could damage cell metabolism might involve the reaction of these free radicals with DNA, and Pryor (1970) has mentioned that hydrogen peroxide is known to alter the chemical structure of adenine. It has also been shown that there was a higher incidence of chromosome aberrations in cells with low catalase activity and high peroxide level. As well as this, many enzymes containing S-S groups have been reported on exposure to light to lose activity, due to a cleavage of the disulphide bonds by radicals, with a consequent formation of -SH groups. One of the most important processes implicated in the chemisty of ageing is that of lipid peroxidation, involving the non enzymatic reaction of unsaturated lipids with oxygen as shown overleaf:-



The participation of lipid peroxidation in membrane damage has been shown in plant tissue (N.Harris, pers.commun.), and unsaturated fatty acids might be in close association with the electron transport system, which in C.diospyri may be flavoprotein in nature. It could then be suggested that, if such a chemical organisation did exist, there might be some protective interaction between lipids, carotenoids and other compounds like those which contain -SH groups within the cell membrane, with one being preferentially peroxidised at the expense of the others. Such an instability of carotenoids has been shown by Blain, (1970). He reported that extracts from a wide range of plant tissue possessed the ability to bleach carotenoid solutions in vitro, and because of the protection given by antioxidants to this destruction, attributed its cause to oxidation by haematins such as peroxidase. It would be of interest to compare levels of malondialdehyde, a compound resulting from the peroxidation of unsaturated lipids (Patton & Kurtz, 1951) in light and dark grown cells of C.diospyri, especially, since from the preliminary work reported here, there appears to be recognisable qualitative differences between separated total lipid extracts from them (Plate X).

The inconclusive nature of some of this work was due in part to the biological material used and the methods employed to produce it. In this contect, it is felt that the opinion expressed by Cantino (1961) is particularly relevant.

"Septate filamentous fungus because of its very nature, and because of the usual methods used for its propagation does not lend itself

readily to studies of this kind. A mass of mycelium started from a few reproductive units and grown for days on solid and liquid medium is a mixture of cells at many states of physiological and chronological age. If the end in view is an understanding of the biology of the individual organism itself, then clearly the filamentous fungus is inherently a difficult creature to deal with, and to hurdle the obstacles that it offers will tax the ingenuity of the experimental biologist. Synchronised cultures of a filamentous fungus would help achieve the solutions to many problems in mycology."

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In this copy the transparent sheet and hence the bracket for the pigmented zone of growth has been misplaced in binding.